# *In vivo* effects of South African traditional medicines against *Mycobacterium tuberculosis* in experimental mice

NCHINYA BENEDICT BAPELA

A thesis submitted to the Department of Pharmacology, University of Cape Town in fulfilment of the requirements for the degree

MASTER OF SCIENCE (MEDICINE)

SUPERVISORS

Prof. B Ryffel

Prof. P Smith

December 2001

# CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# DECLARATION

I, NCHINYA BENEDICT BAPELA, hereby declare that the work on which this thesis is based is my original work and that neither the whole work nor any part of it has being, is being, or is to be submitted for another degree in this or any other University.

I empower the University of Cape Town to reproduce for the purpose of research the whole or any part of the contents in any manner whatsoever.

Signed by candidate

Date: 04-03-2002

# ACKNOWLEDGEMENTS

It is with great appreciation that the following persons are acknowledged:

My supervisors, Professor Bernard Ryffel (Department of Immunology, UCT) and Professor Pete Smith (Department of Pharmacology, UCT) for their time, interest and supervision of this project.

Professor Peter Folb (Head Department of Pharmacology, UCT) for his guidance and advise on this project.

Dr Gilbert Matsabisa (M.R.C) for being a good friend and for sharing of knowledge, encouragement and support wherever it was most needed.

Dr Joseph Mpagi (Department of Immunology) and Dr Paul Waako (Department of Pharmacology) for their advises on writing of my thesis.

Najmeeyah Brown for spending time with me at the animal unit, teaching me the techniques and above all for being such a good friend.

Medical Research Council of South Africa for their financial support.

My family for their support, encouragement, understanding and for enduring so much, especially through the difficult times.

My colleagues, Bongi Gumede, Eliya Madikane, Wendy Dryding, Muazzam Jacobs, Naseema Allie and Siyabulela Ntutela for sharing their knowledge.

Finally, to all the traditional doctors who supplied us with information and my special friend, Teboho Sontaha for making me a dreamer. Thanks so very much for being the wind beneath my wings.

## *DEDICATION*

This thesis is dedicated to my late brother, **Peter Bapela**, for being a positive motivating force in my life and supporting me through the trials and tribulations of life.

# ABSTRACT

Although it is more than 100 years since Robert Koch discovered the tubercle bacillus, and more than 40 years since effective chemotherapy became available, the incidence of tuberculosis is increasing in much of the developing world and has recently re-emerged as a public health problem in industrialized countries. This problem is compounded by the increase in host susceptibility to tuberculosis caused by co-infection with HIV (Human Immunodeficiency Virus) and the emergence of *Mycobacterium tuberculosis* strains that are resistant to the front line drugs. These factors highlight the urgent need for development of new drug classes to counter the threat posed by tuberculosis.

The purpose of the present study was to develop a mouse model for *Mycobacterium tuberculosis* with the aim of determining the antimycobacterial activity of medicinal plants used by traditional doctors to treat tuberculosis in South Africa. Furthermore, the toxic effects of these medicinal plants in uninfected mice were determined.

A field trip to the Northern Cape, Western Cape, Eastern Cape and Free State provinces was undertaken and medicinal plants used by traditional doctors to treat tuberculosis or its symptoms were collected, identified and examined for their therapeutic effects against *Mycobacterium tuberculosis,* determined using the mouse model. In addition, the effects of medicinal plants on the production of cytokines and granuloma formation in infected mice were examined.

Six-to-ten week old C57BL/6 mice were infected with $10^7$ viable *Mycobacterium tuberculosis* H37Rv strain by an aerosol exposure model. Bacterial growth was monitored by sacrificing infected but untreated mice at day 1, week 2 and week 4. Treatment with medicinal plant extracts was started 2 weeks after infection and continued for 2 weeks. An INH-RIF combination was used as positive controls.

The bacterial load in infected but untreated mice increased by 1 log unit each week for 2 to 3 weeks. Bacterial loads were not detected in INH-RIF treated mice after 2 weeks of treatment. Treatment of mice with high doses of plant extracts was toxic. None of the tested medicinal plant extracts showed any activity against *Mycobacterium tuberculosis*. The production of IL-12 at week 4 was suppressed/decreased when plant extract A was given at different concentrations. The bacterial loads in the lungs of the plant extract A treated mice was higher than that of the untreated mice ($p < 0.005$). Histological analysis of the lungs also revealed a high number of bacilli and increased size of the formed granuloma.

In conclusion, the selected plant extracts obtained by water extraction exhibited no anti-tuberculosis activity in the laboratory mouse model for *Mycobacterium tuberculosis* infection. Furthermore, it was also shown that some plant extracts suppressed the production of IL-12, which plays an important role in the host's defense against *Mycobacterium tuberculosis*. However, further work is required to test if treatment for longer periods exhibits antituberculous activity.

# Chapter 1
# LITERATURE REVIEW

## 1.1 INTRODUCTION

Tuberculosis is an infection, often of a lifelong duration, caused by *Mycobacterium tuberculosis*. It can result in disease in virtually every organ system in the body, most prominently the lungs, characterized histologically by granuloma formation (1). The mouse is one of several animal models that can be used in the study of experimental tuberculosis infection. The mouse is cost-effective to use and relatively easy to handle. Like the majority of healthy humans, the mouse is able to generate a strong immune response to *Mycobacterium tuberculosis*. The usefulness of the mouse model has grown in parallel with the explosion of knowledge in the field of immunology. Thus, those studying immunity or immunopathology associated with tuberculosis in the mouse model have profited greatly from the large number of immunological reagents now available. Of these reagents, the great majority are monoclonal antibodies, which made it possible to study the key protein and lipoglycan molecules of the bacillus, to measure cytokines secreted by activated macrophages and T-cells (2).

Current strategies for control of tuberculosis center on treatment with multi-drug regimens based on the very effective combination of Rifampicin and Isoniazid. In endemic areas, the diagnosis and treatment of smear positive patients are emphasized in order to interrupt the spread of the disease within the community. Obstacles to the success of this strategy are the difficulty of early diagnosis and operational problems associated with delivery of a treatment that involves administration of multiple drugs over a period of at least six months (3). Factors that have already compounded these difficulties include increases in host susceptibility to tuberculosis caused by co-infection with human

immunodeficiency virus (HIV) and the emergence of *Mycobacterium tuberculosis* strains that are resistant to the front- line drugs. Therapy for tuberculosis is arduous due to its long duration and multi-drug regimens (3). These phenomena are responsible for the increasing demand for the development of new compounds to treat tuberculosis. Pharmaceutical companies have not perceived the development of new compounds as a high priority for the last 30 years. A co-coordinated effort to screen general medicinal plants for activity against *Mycobacterium tuberculosis* may well prove worthwhile.

In this chapter, epidemiology of tuberculosis globally and in South Africa is discussed and recent advances in understanding the host immune response to tuberculosis together with the pathogenesis of tuberculosis are highlighted. Also discussed are strategies for prevention, control and treatment of tuberculosis based on the first-line anti-tuberculosis drugs.

## 1.2 TUBERCULOSIS

### 1.2.1 EPIDEMIOLOGY

The World Health Organization has rightly declared tuberculosis to be a global emergency because globally the incidence of tuberculosis is rising rapidly (WHO Bulletin, 2001). It is estimated that more than a third of the world population is infected with *Mycobacterium tuberculosis* (Table 1.1). Only about 5% of those infected develop active disease during the first few years following exposure, but this represents 8 million new cases of the disease and almost 3 million deaths occurring annually, the most for any single infectious disease (1).

**Table 1.1 The global toll of tuberculosis**

| Region | People infected (Millions) | New cases | Deaths |
|---|---|---|---|
| Africa | 171 | 1 400 000 | 660 000 |
| Americas* | 117 | 560 000 | 220 000 |
| Eastern Mediterranean | 52 | 594 000 | 160 000 |
| South-East Asia | 426 | 2 480 000 | 940 000 |
| Western Pacific§ | 574 | 2 560 000 | 890 000 |
| Europe and other Industrialized countries¡ | 382 | 410 000 | 40 000 |
| Total | 1 722 | 8 004 000 | 2 910000 |

*Excluding USA and Canada

§Excluding Japan, Australia and New Zealand

¡USA, Japan, Australia and New Zealand

The two essential factors for the rapid spread of tuberculosis are crowded living conditions, which favor airborne transmission, and a population with little natural resistance. Disease due to tuberculosis in populations is influenced by three distinct risks:

- The risk of an individual in the community being infected with tubercle bacilli in a given time period.
- The risk of the disease following shortly after such infection.
- The risk of the disease occurring long after the original infection owing to the reactivation of latent bacilli (4).

South Africa is burdened by one of the worst tuberculosis epidemics in the world, with disease rates more than double those observed in other developing countries and up to 60 times higher than those currently seen in developed countries. The tuberculosis problem in South Africa is largely a result of

fragmented health services (5). In South Africa, a high proportion of the population lives under poor conditions and this may lead to the burden of the disease becoming uncontrollable. Estimates by the Medical Research Council's (MRC) National Tuberculosis Research Programme (TBRP) put the burden of tuberculosis for the year 2000 at 273 365 new cases, of whom 113 945 were to be infectious and 46,7 would also be HIV positive (Table 1.2). In the year 2000 South Africa was also expected to contribute at least 15% of the total tuberculosis caseload for Africa. The burden of tuberculosis is not distributed evenly throughout South Africa and rates vary considerably among the nine provinces (Fig 1.1).

Fig 1.1 Tuberculosis rates in the 9 provinces of South Africa

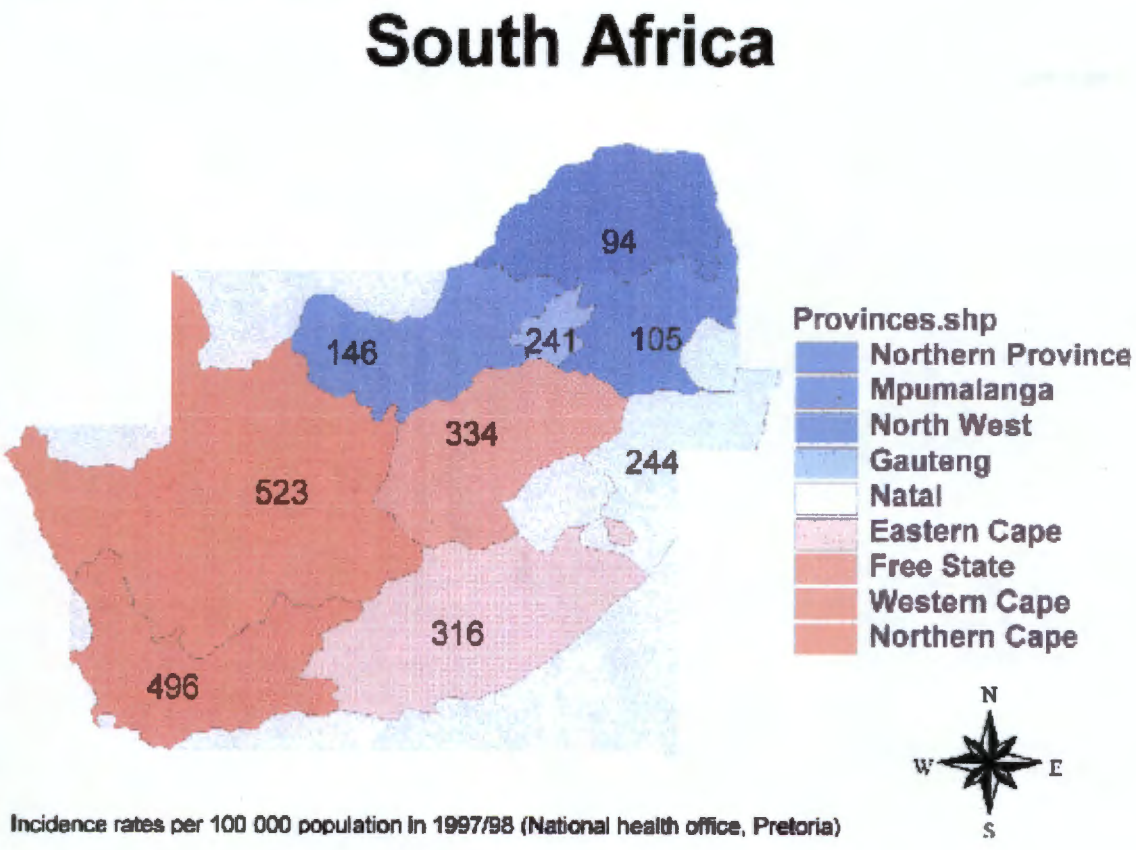


**Table 1.2 Annual tuberculosis burden for South Africa: 600 total cases per 100 000 population; 250 smear-positive cases per 100 000 population.**

| Province | Total TB cases | Proportion HIV+ |
|---|---|---|
| Northern Province | 23 338 | 36.3% |
| Mpumalanga | 15 657 | 59.1% |
| North West | 15 549 | 45.5% |
| Gauteng | 45 598 | 44.8% |
| Free State | 14 654 | 51.7% |
| Kwazulu-Natal | 65 695 | 64.6% |
| Eastern Cape | 56 495 | 40.0% |
| Northern Cape | 4 649 | 33.2% |
| Western Cape | 34 211 | 31.6% |
| South Africa | 273 365 | 47.6% |

## 1.2.2 TRANSMISSION OF TUBERCULOSIS

The principal risk of acquiring infection with *Mycobacterium tuberculosis* is breathing (1,4). Tuberculosis causing microbes are present in sufficient concentrations in the air to cause infections and disease. Central to the concept of airborne transmission is the droplet nucleus, a microscopic particle 1 - 5 microns in size, consisting of no more than 2 or 3 viable bacteria surrounded by a layer of moisture. Once in air, water evaporates from the surface of the particle, decreasing its size and concentrating its contents of microbes.

These particles form droplet nuclei in which evaporation continues until the vapor pressure of the droplet equals the atmospheric pressure. The droplet nuclei are very stable, settle very slowly and remain suspended in the air for very long periods. Droplet nuclei are produced when a patient with active pulmonary or laryngeal tuberculosis coughs, speaks, sneezes or sings. Coughing can produce 3000 infectious droplet nuclei, talking for 5 minutes an equal

number and sneezing can produce over a million particles with a diameter of less than 100nm (Riles et al, 1961).

Breathing on its own does not produce these particles. When breathed in, droplet nuclei are not deposited on the walls of the upper airways, trachea or bronchi, they remain suspended in the airway until they reach the alveoli. Larger particles that are deposited on the airway are removed through normal mechanism of airway clearance. Factors determining the likelihood of transmitting tuberculosis infection are:

- **Number of organisms**

When many infectious bacillary particles are inhaled, a phenotypically hardy bacillus is likely to be among them. Pulmonary infection would begin only after a strongly endowed bacillus is ingested by a weakly endowed alveolar macrophage. No one knows exactly how many particles containing tubercle bacilli must be inhaled before such a combination is reached; for human beings, the figure is probably between 5 and 200 (6).

- **Virulence of organisms**

Tubercle bacilli may, both genetically and phenotypically, vary in virulence. The virulence of human-type tubercle bacilli (H37Rv) can sometimes be reduced by repeated sub culturing, indicating that its genotype is not completely stable. A viable bacillus that is dried out or exposed to sunlight will often be phenotypically too weak to initiate an infection. A viable bacillus kept moist in a dark place will be more infectious when aerosolised (6).

## 1.2.3 PATHOGENESIS

Airborne droplet nuclei containing very few or even single infectious units are small enough to avoid entrapment by the bronchial mucociliary apparatus and reach the terminal air space where multiplication begins. The heavier bacillary particles (containing more bacilli and/or bits of caseous material) impinge upon the mucosal surfaces of both the nasopharynx and the bronchial tree, are moved along the cilia, and are eventually swallowed or they are coughed up and destroyed (6).

The initial focus is usually subpleural in location and usually located in the midlung zone where greater airflow favors deposition of inhaled bacilli. The inhaled bacillus reaches the alveoli and is ingested by an alveolar macrophage. In such macrophages the bacillus can be destroyed or inhibited or can multiply intracellularly. If the bacillus multiplies, the alveolar macrophage dies and it's bacillary load are ingested by other alveolar macrophages emigrating from the pool of circulating monocytes. However, bacterial multiplication tends to be mostly unimpeded, eventually destroying the macrophages. The tubercle bacilli multiply within tissue macrophages in a state of symbiosis, where most of the intracellular bacilli and most of the macrophages containing them live in apparent harmony. Logarithmic growth of the bacilli occurs from day 7 to day 21 (7).

In 3 - 4 weeks however the immune system responds. This is characterized by the onset of Cell Mediated Immunity and Delayed Type Hypersensitivity. Alveolar macrophages now lymphokine activated by T- lymphocytes, demonstrate an increased ability to destroy intracellular bacilli. As a result, the logarithmic increase in the number of organisms ceases. At the same time bacilli kill macrophages and their bacillary load is often ingested by nearby macrophages. These cells in turn will die if the bacilli multiply within them. A

tubercle forms that consists of a caseous necrotic center surrounded by granulation tissue containing viable macrophages, lymphocytes and other cell types.

The caseous center will then grow, and the lesion will enlarge. Liquefaction of the caseous centers of tubercle is one of the most harmful host responses in tuberculosis. Liquefaction seems to be due to hydrolysis of the protein, lipid and nucleic acid components of caseous tissue by the hydrolytic enzymes in the macrophages. Liquefaction perpetuates tuberculosis in humans. The liquefied caseum is an excellent culture medium for the tubercle bacillus. In this environment, the bacillus extensively multiplies extracellularly for the first time and attains large numbers. This large antigenic load is toxic to tissues. The walls of the nearby bronchi often become necrotic and rupture, forming a cavity. Delayed Type Hypersensitivity continues to the point of cavity formation and destruction of cavity walls. The bacilli and bits of liquefied and caseous tissue are then discharged into the airway reaching other parts of the lung and the outside environment (6,7).

### 1.2.4 IMMUNOLOGY OF TUBERCULOSIS

Tuberculosis is the prototype of infections that require cellular immune response for their control. In the first few weeks the host has almost no specific immune defense against infection by *Mycobacterium tuberculosis*. Small inhalation inocula multiply freely in the alveolar space or within alveolar macrophages. Unrestrained bacterial multiplication proceeds until the development of tissue hypersensitivity and cellular immunity supervene.

*Mycobacterium tuberculosis* adheres to alveolar macrophages via multiple complement receptors (CR1, CR3, CR4, etc) and then internalized into a phagosome where it may be killed (8). The intracellular mechanisms for killing or inhibiting growth of *Mycobacterium tuberculosis* in alveolar macrophages include the production of nitric oxide (NO) and reactive oxygen intermediates (ROI) as well as apoptosis induced by H2O2 or cytotoxic T-cells. Alveolar macrophages can also participate in a broader context of cellular immunity through the process of antigen presentation and recruitment of T-lymphocytes.

Macrophages present antigens processed in phagosomes via major histocompatibility complex (MHC) class 11 molecules to CD4 T-lymphocytes, the major effector cells in cell-mediated immunity. The antigens bind to T-cell receptors on the surface of the T-lymphocytes. These CD4 T-lymphocytes tend to polarize into Th1 cells producing predominantly interferon gamma (IFN-γ) and interleukin 2(IL-2) or Th2 cells producing predominantly IL-4, IL-5, IL-6, IL- 10 and IL-13. In mice, immunity correlates with a Th1 response. Macrophages infected with *Mycobacterium tuberculosis* secrete IL-12, which induces the secretion of IFN-γ by CD4 cells and natural killer cells. The IFN-γ enhances the activation of macrophages and improves their ability to contain the spread of *Mycobacterium tuberculosis* (2,7,8). However *Mycobacterium tuberculosis* is not defenseless. It can produce ammonia to counteract phagosomal acidification. Its lypoglycan coat (LAM) can actively scavenge toxic radicals produced against it by the macrophage as well as produce enzymes such as superoxide dismutase to inactivate radicals.

## 1.2.5 TREATMENT OF TUBERCULOSIS

Before effective drugs were available, half of the patients with active pulmonary tuberculosis died within 2 years, and only a quarter were cured. With the advent of chemotherapy, protracted bed rest, lengthy isolation, and collapse therapy became unnecessary, and in theory at least, successful treatment was a reasonable goal in all adults (9,10).

Current therapy for pulmonary tuberculosis involves 6 months of treatment with Isoniazid, Pyrazinamide, Rifampin and Ethambutol or Streptomycin for reliable treatment therapy. The long treatment therapy increases the probability of non-compliance, leading to the generation of multidrug-resistant isolates of *Mycobacterium tuberculosis*. Current therapies reduce the pulmonary bacterial burden, but treatment periods of 6 months for nonimmunosuppressed individuals and at least 9 months for immunosuppressed patients are required for reliable treatment efficacy (4, 10).

- **ISONIAZID (INH)**

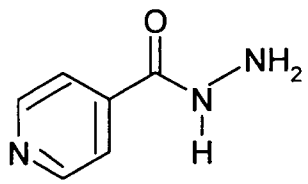



Fig 1.2. Structure of Isiniazid

INH is the cornerstone of therapy and should be included in all regimens unless a high degree of INH resistance exists. INH acts almost exclusively against *M. tuberculosis, M. bovis* and *M. africanum*. INH is highly selective. This remarkable selectivity in its action is thought to be mediated by the bacterial enzyme catalase peroxidase which catalyses the reaction converting INH to a potent bactericidal derivative. INH is bactericidal at minimal inhibitory concentration (MIC) levels of less than 0.1 g/ml for 80% of susceptible strains of *M. tuberculosis* (10).

## MECHANISM OF ACTION

INH may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the infecting organism. The exact mechanism of action of INH has not been fully elucidated, but several mechanisms including interference with metabolism of bacterial proteins, nucleic acids, carbohydrates, and lipids have been proposed. One of the principal action of the drug appears to be inhibition of mycolic acid synthesis in susceptible bacteria, which results in loss of acid-fastness and disruption of the bacterial cell wall. INH is bacteriostatic for "resting" bacilli but is bactericidal for rapidly dividing microorganisms. Susceptible bacteria may undergo 1 or 2 divisions before multiplication is arrested.

## TOXICITY

The hepatotoxicity associated with INH result from the toxic effect of an intermediate product produced by N-hydroxylation of monoacetylhydrazine, one of the metabolite of INH, by the liver cytochrome P-450 mixed function oxidase system (Holdiness et al, 1984). Mice are most sensitive to INH and, surprisingly, the method of administration has scarcely any effect on toxicity in this species (10).

- **RIFAMPICIN (RMP)**

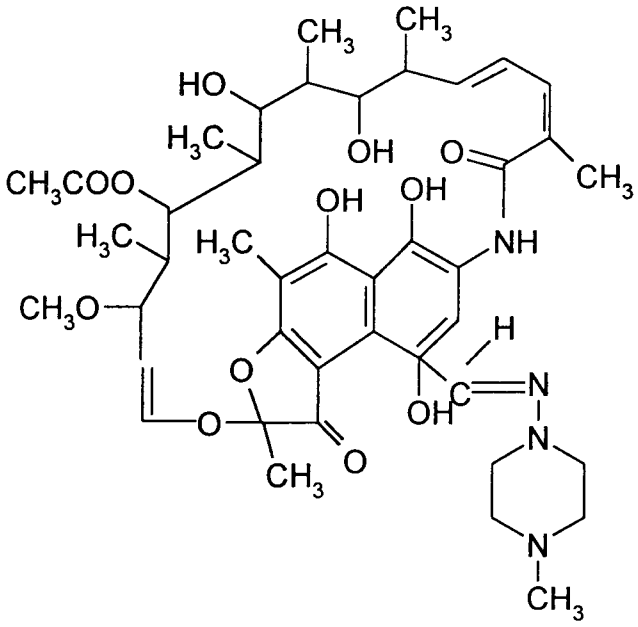



Fig 1.3. Structure of Rifampicin

Rifampicin is the second major antituberculous agent. RMP is a semi synthetic derivative of one of a group of structurally similar, complex macrocyclic antibiotics produced by *Streptomyces Mediterranei.* RMP inhibits the growth of most gram-positive bacteria as well as many gram-negative microorganisms. RMP in concentrations of 0.005 - 0.2 g/ml inhibits the growth of *M. tuberculosis* in vitro. RMP is soluble in organic solvents and in water at acidic pH. It is used in conjunction with other antituberculosis agents in the treatment of clinical tuberculosis.

**MECHANISM OF ACTION**

RMP inhibits DNA-dependent RNA polymerase of mycobacterium and other microorganisms by forming a stable drug-enzyme complex, leading to suppression of initiation of chain formation (but not chain elongation) in RNA synthesis. More specifically, the subunit of this complex enzyme is the site of action of the drug, although RMP binds only to the holoenzyme. Nuclear RNA polymerase from a variety of eukaryotic cells does not bind RMP, and RNA synthesis is correspondingly unaffected. While RMP can inhibit RNA synthesis

in mammalian mitochondria, considerably high concentrations of the drug are required than for the inhibition of the bacterial enzyme. RMP is bactericidal for both intracellular and extracellular microorganisms (10,11,12).

### TOXICITY

In humans acute overdose with RMP doses up to 12g have not been fatal, at least one fatality has been reported following ingestion of a single 60g dose of the drug. The lethal dose (LD50) of RMP in mice is 0.885g/kg. The most important complication of RMP is Hepatitis. This occurs 4 times more frequently in regimens containing both INH and RMP than those containing INH alone.

## • PYRAZINAMIDE (PZA)

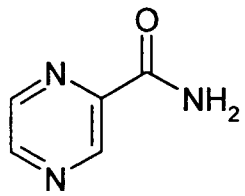



Fig 1.4. Structure of Pyrazinamide

PZA a derivative of niacinamide, is a synthetic antituberculosis agent. PZA is active against tubercle bacilli, perhaps because it possesses an enzyme pyrazinamidase, which transforms PZA into pyrazinoic acid believed to be intracellularly active substance. Currently, PZA is considered a first line agent and is the third most important drug used in the treatment of tuberculosis. Its beneficial effect is mostly limited to the first 2-4 months of treatment.

### MECHANISM OF ACTION

PZA may be bacteriostatic or bactericidal in action, depending on the concentration of the drug attained at the site of infection and the susceptibility of the infecting organism. In vitro and in vivo, the drug is active only at a slightly acidic pH. The exact mechanism of action of PZA has not been fully elucidated. The antimycobacterial activity of PZA appears to partly depend on conversion of

the drug to pyrazinoic acid (POA). Susceptible strains of *M. tuberculosis* produce pyrazinamidase, an enzyme that deaminates pyrazinamide to POA, and the in vitro susceptibility of a given strain of the organism appears to correspond to its pyrazinamidase activity. In addition, the fact that POA lowers the pH of the environment below that which is necessary for *M. tuberculosis* growth appears to contribute to the drug's antimycobacterial activity in vitro (11,12).

**TOXICITY**

The most frequent adverse effect of PZA is hepatotoxicity. Hepatotoxicity is a problem when PZA is given in larger doses and for longer periods. Hepatotoxicity may appear at any time during therapy. When it is used in short-course therapy no increase in the incidence of hepatotoxicity is noted. In mice PZA has a LD50 of 3.4g/kg following oral administration (Robinson et al, 1954). Higher doses results in excitation, rapidly followed by depression.

- **ETHAMBUTOL (EMB)**

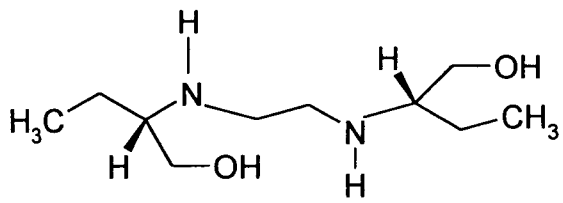



Fig 1.5. Structure of Ethambutol

EMB is a synthetic antituberculosis agent. EMB is a highly specific agent and is active only against organisms of the genus *Mycobacterium*. It is active in vitro and in vivo against *M. tuberculosis, M. bovis, M. marinum* and some strains *of M. kansasii, M. avium* and *M. intracellulare*. EMB is usually given at a daily dose of 25mg/kg during the first 2 months of well-supervised therapy and at 15mg/kg for longer often less well-supervised periods.

### MECHANISM OF ACTION

EMB is bacteriostatic in action. Although the exact mechanism of action has not been fully elucidated, the drug appears to inhibit the synthesis of one or more metabolites in susceptible bacteria resulting in impairment of cellular metabolism, arrest of multiplication, and cell death. EMB is active against susceptible bacteria only when they are undergoing cell division (12).

### TOXICITY

A single administration of EMB has only a low toxicity in mice (Diermeier et al, 1966). In humans adverse effects of EMB include dermatitis, pruritus, headache, dizziness, fever and mental confusion among other things.

## 1.2.6 PREVENTION OF TUBERCULOSIS

The outcome of mycobacterial infection critically depends on the host immune response. In most individuals, infection with *Mycobacterium tuberculosis* induces an immune response sufficient for protection against progression to primary disease. BCG vaccine reproduces as near as possible the initial natural infection but does not impose a disease risk. BCG vaccine, which is derived from a strain of *M. bovis* attenuated through years of serial passage in culture, was first used in 1921 to protect against tuberculosis in human (3). Many BCG vaccines are available worldwide; all are derived from the original strain but vary in culture characteristics and in ability to induce sensitization to tuberculin. BCG vaccines vary because of genetic changes in the bacterial strain and because of differences in techniques of production, in methods and routes of vaccine administration.

BCG vaccine is currently administered to 100 million young children each year throughout much of the world. This is to protect children against primary and disseminated forms of tuberculosis. That is why it should be administered only to tuberculin-negative persons. While BCG vaccine does not prevent infection, it usually prevents progression to clinical disease, and its effectiveness in preventing disseminated disease in children is striking (3). BCG should not be given to persons with established HIV infection.

## 1.2.7 CONTROL OF TUBERCULOSIS

The WHO-recommended treatment strategy for detection and cure of tuberculosis is Directly Observed Treatment System (DOTS). DOTS combines five elements: political commitment, microscopy services, drug supplies, surveillance and monitoring systems and use of highly efficacious regimes with direct observation of treatment. Once patients with infectious tuberculosis have been identified using microscopy services, health and community workers and trained volunteers observe and record patients swallowing the full course of the correct dosage of anti-tuberculosis medicines. Sputum smear testing is done after 2 months, to check progress, and again at the end of treatment. A recording and reporting system documents patients' progress throughout, and the final outcome of treatment.

**General strategies for controlling TB**

- Early identification and treatment of persons with infectious tuberculosis. This does not only cure the affected person but also renders the patient non-contagious within a few weeks.
- Identifying and treating persons with non-contagious tuberculosis. This is useful in preventing progression of infection to clinical disease.

- Use of ventilation and ultraviolet lights to decontaminate air containing infectious droplet nuclei. This is used routinely only where the risk of transmission is known to be exceptionally high.
- Vaccination of uninfected children who are at unavoidable risk of exposure to tuberculosis and for whom other methods of prevention and control have failed or are not feasible.

In South Africa some progress is being made in certain provinces. Mpumalanga and the Western Cape are already showing dramatic improvements in cure rates, because of disciplined implementation of the DOTS strategy of the WHO. Other provinces are at various stages of implementation of the process (5).

## 1.3 CONCLUSION

Increased knowledge of current risks of infection and subsequent disease could help greatly in efforts to bring tuberculosis back under control and, in developed countries, could even lead to its elimination in the near future.

Progress towards understanding of immune response to mycobacterium has accelerated in the last decade, but it remains for this progress to be translated into practical advances relevant to the control of tuberculosis. New understanding of the immunology of tuberculosis offers a promise of new and advance modalities. It is reasonable to expect the pace of progress to continue to increase, driven by the magnitude of the global problem compounded by the pandemic of HIV infection and outbreaks of multidrug-resistant tuberculosis.

The human immune response to *Mycobacterium tuberculosis* is a complex reaction that is both beneficial and deleterious to the infected individual. It

requires the interaction of several types of cells that cross-regulate each other's activities via mixture of soluble cytokines. The latter often must act in concert or sequentially to stimulate a given cellular function and may have opposing effects on different cell types. Advances in basic biology now render it possible to directly analyse immune response in infected or diseased human tissues and body fluids. This approach should permit a reappraisal of concepts based on older models and improve our understanding of the pathogenesis of human tuberculosis infection and disease.

Adherence to medical treatment depends on the characteristics of the treatment, the characteristics of the health-care delivery system, and the patient/health-care worker bond. Having bilingual and bicultural staff if the patient population speaks languages other than the dominant one enhances the relationship between the patient and all members of the tuberculosis-control program team.

A better understanding of the biology of *Mycobacterium tuberculosis* will hopefully address such issues as developing medications or modulators that can kill the organism in the actively and intermittently metabolizing, as well as dormant phases, developing medications that will both shorten treatment and allow it to be given intermittently, as was the case with Rifampin, activating dormant organisms so that drugs can work against them, and determining how to better prevent drug resistance.

# CHAPTER 2
# TRADITIONAL MEDICINE

## 2.1 INTRODUCTION

The use of plants or plant extracts for medicinal purposes has been going on for thousands of years, and herbalism and folk medicine, both ancient and modern, have been the source of much useful therapy (13).

Traditional medicine has been practiced for at least seven thousand years. To this day, the majority of the world's populations receive their health care from traditional medicinal systems. The practices are based on beliefs that were in existence before the development and spread of modern scientific medicine, and that are still prevalent today. Traditional medicine is part of a country's cultural heritage handed down from generation to generation. Experimentation with plants and the passage of knowledge from one generation to the next resulted in the development of a vast knowledge about plants for use as foods, medicines and their method of preparation (13,14). Millions of people worldwide still depend upon traditional therapies that are based on medicinal plants, often administered in very specific ways under the direction of a knowledgeable healer. Plants have long been used among indigenous South Africans as remedies against infectious diseases. It is estimated that 70 - 80% of the indigenous people of South Africa still consult traditional healers for curing their diseases (14). Many plant preparations have been used externally as disinfectants and antiseptics for wounds and pimples, as antidiarrhoeics and in the treatment of respiratory diseases (15).

Tuberculosis is a leading contender for the dubious distinction of being the most important plague to humankind. Tuberculosis has also been termed as the "disease of the poor". This is because a large proportion of tuberculosis incidences come from countries of poor economic status and associated malnutrition. A large proportion of them rely on traditional means of health. Among the many forms of treatment they use are herbal preparations. Many of these herbal medicines have been used from generation to generation with great confidence of efficacy (8)

In this chapter, collection of plants from the field trip is discussed. Furthermore, perceptions and understanding of tuberculosis by traditional doctors is discussed. Methods of diagnosis and treatment procedures by traditional doctors are also mentioned.

## 2.2 METHODS

### 2.2.1 PLANT COLLECTION

We consulted traditional healers, herbalists and Sangomas (Traditional doctors) on the plants they use to treat tuberculosis or chest problems. The plants were then either bought from them or collected in the field with their help. Information on the part of the plant used, how it is prepared, how it is administered and how much is taken was noted. Traditional names of plants were given in almost all cases and their botanical names were checked/confirmed on the traditional medicine database at the Department of Pharmacology, UCT.

### 2.2.2 PLANT PREPARATION

The collected plant material was air-dried at room temperature. The dried material was ground using the laboratory blender (Waring products, U.S.A) to

increase the surface area of the plant for extraction. The ground plant material was mixed the same way traditional doctors prepare their remedies.

Table 2.1 Plants used for tuberculosis

| Symbol | Vernacular name | Botanical name | Part used |
|---|---|---|---|
| A | Wynruit | Ruta graveolens | Leaves |
| | Sieketroos | Arctopus echinatus | Root |
| | Varkoor | Cotyledon orbiculata | Leaves |
| B | Iphuzi | Gunnera perpensa | Root |
| | Umagaqana | Croton sylvaticus | Tuber |
| C | Undawuluthi | Sparaxis grandiflora | Tuber |
| | Isibhara | Warburgia ugandensis | Root |
| | Ubuvimba | Withania somnifera | Root |
| D | Rooiwortel | Bulbine natalensis | Root |
| | Bergsalie | Buddleia salvifolia | Leaves |
| | Koorsbos | Dicoma capensis | Leaves |
| E | Iqwili | Alepidea prunelloides | Root |
| | African potato | Hypoxis hemerocallidea | Tuber |
| F | Varkoortjies | Centella asiatica | Leaves |
| G | Inqwebeba | Ledebouria revoluta | Root |
| | Mathonga | Eucomis autumnalis | Bulb |
| H | Isicimamlilo | Pentanisia prunelloides | Root |
| I | Isibhara | Warburgia ugandensis | Root |
| J | Umdlavuza | Eucalyptus qlobulus | Bark |
| | Umkhwenkhwe | Pittosporum viridiflorum | Bark |
| K | Iphuzi | Gunnera perpensa | Root |
| | Iqwili | Alepidea prunelloides | Root |

### 2.2.3 EXTRACTION

Known amount of the plant material was extracted using water as a solvent. Hot and cold extraction of the plant material was carried out for 24 hours each, followed by filtration of the extracts using a Whatman filter paper (0.15cm, Millipore, Bedford, MA). Cold extraction was carried out on the shaker (Labcon, labdesign engineering, Maraisburg) and hot extraction on the Soxhlet apparatus (Appendix A). The filtrates were concentrated by freeze-drying the water component on the freeze-drier and the yields calculated. The extracts were stored in the freezer at $4^0$C until use.

### 2.2.4 FIELD TRIP

Before starting the project a field trip was undertaken to the Northern Cape, Free State, Eastern Cape and the Western Cape provinces (Fig 2.1). In the Northern Cape we were interested in knowing how the Khoisan people treat tuberculosis in their communities. This is because little information is known about their traditional healing systems. Khoisan people use small trees to treat diseases because they live in a semi desert place where there are no big trees. In other regions, barks and leaves from big trees are used in the treatment of tuberculosis. We also found that in different regions different plants were prescribed for the treatment of tuberculosis. It was not possible, however, to record the response of the medicinal plant therapy as patients were not readily available for interview at the time of the visits. Moreover, traditional doctors have no organized hospitals and the sick that receive treatment are always outpatients.

# SOUTH AFRICA

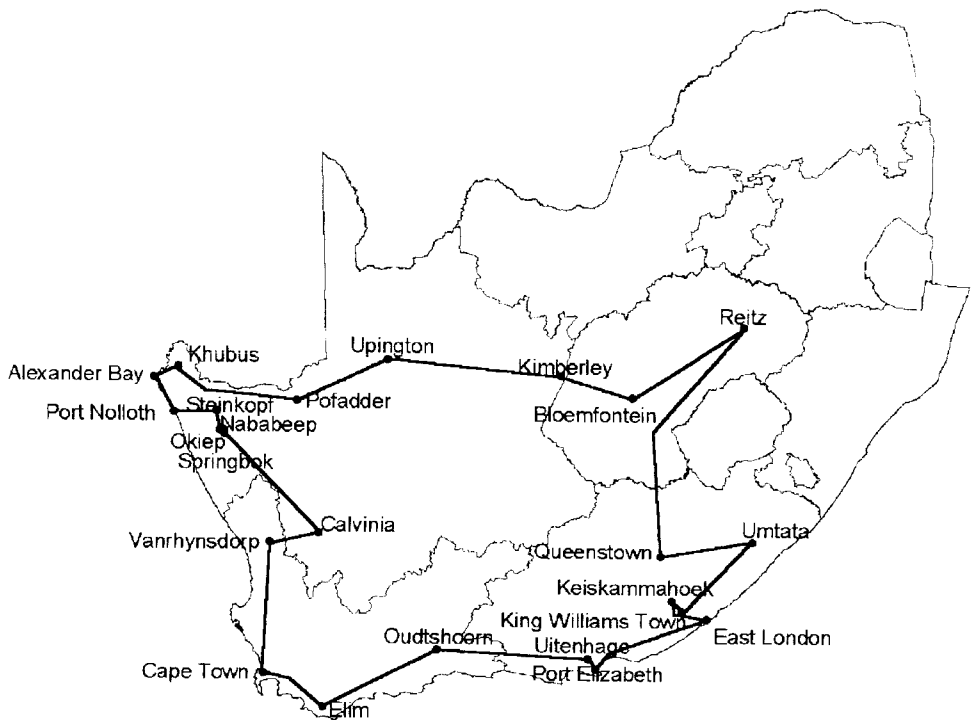



Fig 2.1 The route of the field trip. The trip took 2 weeks and the plant material was either bought or collected in the field with the help of the traditional doctor.

### 2.2.5 PERCEPTION AND UNDERSTANDING OF TUBERCULOSIS BY TRADITIONAL DOCTORS

According to Traditional beliefs tuberculosis is a man made disease. A 'big cough' according to most healers is caused by sorcery. This is practised in order to inflict harm upon some specific enemy, for instance as a response to a grievance in a relationship. The person causing the disease does this by burying bad medicine in the ground and when the victim steps on it will penetrate the foot and cause a disease such as tuberculosis or by giving the victim 'poisoned' food to eat which will cause a disease such as tuberculosis in the body.

Once the bad medicine or 'poisoned' food is in the body, it will go to the lungs where it will cause an internal growth because of 'germs' from it. The germs lead to coughing and weight loss. Like western doctors, traditional doctors also believe that this kind of disease is transmissible and the person suffering from this should always use a handkerchief when blowing his/her nose or hold it in front of his/her mouth when coughing.

### 2.2.6 DIAGNOSIS AND TREATMENT OF TUBERCULOSIS BY TRADITIONAL DOCTORS

When a patient visits a traditional doctor for consultation on a specific disease, the traditional doctor will throw bones to communicate with the ancestors in order for them to show him/her the cause and the solution to a specific disease. During this process, the traditional doctor will advise the patient as to the presenting problem and the reasons or dynamics underlying the malady. The ancestral spirits will then tell them which plant they have to use to treat a particular problem of a patient and where they can find the plant. This is then followed by advice referring to a treatment approach. In most cases, this includes prescribing a herbal remedy.

Treatment is most often directed both to the body and to the mental state, considered in the context of social environment, ancestral spirit, family and community. The treatment chosen is not only symptomatic, it relates to the evolution of the disorder. This in many cases involves giving different herbal preparations for the symptoms as well as the cause of the health problem. One remedy can also contain different plants with different effects. Some traditional doctor's reported that they send some patients to nearby hospitals to confirm or to help them in their diagnosis.

### 2.2.7 CURRENT RESEARCH IN TUBERCULOSIS AND MEDICINAL PLANTS

With the urgent need for new antimycobacterials, South African researchers are now beginning to find safer, rapid and effective ways of assaying plants against *Mycobacterium tuberculosis*. Although no marketable products for the treatment of tuberculosis have been isolated from plants, some lead molecules have been isolated (16). In structure-activity related studies against *Mycobacterium tuberculosis*, many schools of thought have been suggested. Different research groups have found activity on a variety of natural products with no certain trend towards a specific group of compounds. Focus is also paid to the marine-derived natural products, although these are not necessarily of the phytoplankton family (17).

## 2.3 RESULTS

Table 2.2 Yields of plant extractions

| Extract | Starting mass (g) | Final mass (g) | Percentage yield (%) |
|---|---|---|---|
| A | 13.67 | 1.59 | 11.63 |
| B | 5 | 1.70 | 34.00 |
| C | 6 | 1.42 | 23.66 |
| D | 15 | 2.27 | 15.13 |
| E | 10 | 2.24 | 22.40 |
| F | 20 | 3.76 | 18.80 |
| G | 50 | 5.24 | 10.48 |
| H | 10 | 1.23 | 12.30 |
| I | 10 | 2.65 | 26.5 |
| J | 10 | 2.74 | 27.40 |
| K | 5 | 0.90 | 18.00 |

## 2.4 DISCUSSION

South Africa is a country where majority of people still live below the breadline level. The first person to be exposed to these poor people when they get sick is the local traditional doctor. This is because of the traditional doctor's convenient hours and the more personalised service, their offer of a wide variety of curative services and because they are more accessible to the general population. Although not part of the modern health care system in our country, traditional doctors perform a significant role in our society, especially in the rural areas. There should be strengthening of links between modern and traditional medicine practices with the emphasis on improving mutual understanding especially about the practices and techniques of the traditional practitioners.

# CHAPTER 3

## DETERMINATION OF THE THERAPEUTIC AND TOXIC EFFECTS OF MEDICINAL PLANTS

### 3.1 INTRODUCTION

South Africa is witnessing an explosion in the number of tuberculosis cases because of the AIDS pandemic. It is essential to have new antituberculosis agents, preferably those that can be readily and simply produced from some local source. The use of antimicrobials from natural vegetation has a great impact on human health care in undeveloped countries (18). Traditional doctors use a mixture of medicinal plants to treat tuberculosis or its symptoms. In South Africa some patients, especially from rural areas might use these medicines for treatment of tuberculosis. Although these medicines are used extensively to treat tuberculosis or its symptoms, there is little or no information on their toxic effects. Thus, it is important to determine the toxic and therapeutic effects of these medicines. The importance in identification of herbal molecules lies not only in their pharmacological or chemotherapeutic effects but also in their role as template molecules for the discovery of new drug substances.

In this study, plants used to treat tuberculosis or its symptoms by traditional doctors were investigated for their therapeutic effects against *M. tuberculosis* in a mouse model. Rifampin and Isoniazid are both first-line drugs for use in the therapy of tuberculosis and are included in the list of recommended drug regimens for treatment of latent *M.tuberculosis* infection in adults. As a part of this study, we decided to use them as positive controls.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Mice

In all experiments, six to eight weeks old C57BL/6 wild type mice were used. Mice were bred and maintained under specific pathogen free (SPF) conditions in the animal unit at the University of Cape Town (UCT), South Africa. The animals were housed in filter-top cages (5 per cage) and were given food and water *ad libitum* throughout the study. Mice were allowed to acclimatize to their new environment for 3 – 5 days prior to infection.

### 3.2.2 PREPARATION OF INFECTIOUS *MYCOBACTERIUM TUBERCULOSIS*

#### 3.2.2.1 Cultivation

*Mycobacterium tuberculosis* H37Rv was obtained from Department of Immunology, UCT, Cape Town. A small amount of colony was removed from a Lowenstein-Jensen slant using a sterile plastic disposable inoculating loop, and deposited into a 50ml, 25cm$^2$ sterile screw-capped tissue culture flask (Greiner Labortechnik) containing 10ml of Middlebrook 7H9 Broth [Difco]/ 10% OADC (oleic acid-albumin-dextrose-catalase) enrichment media (State Vaccine Institute, Cape Town). Culture flasks were incubated at 37$^0$C in a $CO_2$ incubator and shaken daily to gently agitate the broth. Incubation proceeded for 1-2 weeks until the culture turned turbid (CFU density of 1 X $10^7$/ml) and in the mid-late log growth phase. From this culture 20ml was carefully transferred into a 250ml sterile screw-capped culture flask (Greiner Labotechnik) containing 90ml Middlebrook 7H9 Broth [Difco]/ 10% OADC enrichment medium. Culture flasks containing 100ml of broth were further incubated for 1-2 weeks at 37$^0$C in a $CO_2$ incubator and shaken daily until a similar turbidity was attained. Aliquots of these mid-late log phase cultures were aseptically prepared by pipetting 1ml per vial into sterile 2ml serum vials (Greiner Labotechnik). These aliquots were placed into a labelled storage box within a -70$^0$C freezer until further use. An

placed into a labelled storage box within a -70$^0$C freezer until further use. An aliquot (2ml) of the bacilli culture was plated on the blood agar and incubated overnight to screen for any contamination.

**3.2.2.2 Inoculum determination - Colony Forming Units (CFU)**

The concentration of the bacilli was determined by making 10-fold serial dilutions in 0.9% NaCl/0.04% Tween 80, and plating out dilutions of $10^{-2}$ to $10^{-7}$ on Middlebrook 7H10 Agar (19g 7H10 Agar/1L [Difco], 5ml glycerol, 10% OADC) on 2 compartment plates (90mm plates, Sterilin). These were incubated at 37$^0$C for 15-18 days and colonies counted using an inverted microscope (Wild M8 Heerbrugg).

### 3.2.3 AEROSOL INFECTION OF MICE

**3.2.3.1 Preparation of Inoculum for Aerosol Infection**

An aliquot of *Mycobacterium tuberculosis* was thawed at room temperature, spun down at 4 000 rpm (revolutions per minute) for 5 minutes, the supernatant discarded and the pellet resuspended 30 times using a 1ml syringe fitted with 26.5 gauge needle (Sterilin) in a total volume of 6ml 0.9% saline to disrupt clumping. To obtain the desired rate of infection, the inoculum was prepared 5 $\log_{10}$ CFU higher than what one requires delivered into the lungs of the infected mice (the aerosol machine is programmed to deliver into the lungs approximately 5 $\log_{10}$ CFU lower than the inoculum nebulised) i.e. To obtain 100 CFU/lung, the mycobacterial suspension was prepared at 2.0 X $10^6$ CFU/ml in a total volume of 6ml sterile 0.9% saline (1.0 X $10^7$ CFU/5ml nebulised). To confirm the CFU/ml titer 10-fold serial dilutions of the inoculum from $10^{-1}$ to $10^{-7}$ were plated in duplicates on the Middlebrook 7H10 agar [Difco].

### 3.2.3.2 Aerosol procedure

The aerosol exposure chamber (Middlebrook) was sterilized prior to use with a 3% virkon solution followed by 70% ethanol. The metal support plate and the mesh exposure basket were placed into the chamber drum. The divisions within the drum were labelled with the sex of the mouse and the mice transferred from their cages into the basket. The basket lid was secured and the perspex chamber lid sealed by firmly snapping the latches. After sealing the chamber, the glass Nebuliser-Venturi was mounted to the compressed air and main airflow fittings, which was secured by 3 clamps. A safety mask was placed over the face for the rest of the procedure. Carefully, screw cap on the nebuliser was removed and 5ml of the inoculum added into the bottom of the venturi using a 5ml syringe fitted with an 18-gauge needle (Sterilin). After screwing the lid closed, the instrument was switched on.

The program to be entered for aerosolizing mice was as follows

1. Preheating time = 15 minutes
2. Nebulising time = 40 minutes
3. Cloud decay = 40 minutes
4. Decontamination = 15 minutes

The preheating time is to enable the incinerator to achieve its core temperature of 790$^0$C in order to decontaminate the exhaust air. During the nebulisation cycle the compressed air is activated and passes though the venturi to the nebuliser to create droplet nuclei containing bacilli. These droplet nuclei are carried into the chamber containing the animals. The third cycle is "cloud decay" in which the aerosol chamber is purged with fresh air and the bacterial mist dissipated. During the final cycle the UV lamps are switched on to decontaminate the top surfaces of the basket. Once the instrument had initiated the preheat cycle, the main air flowmeter should indicate 60 cubic feet/hour (CFH), and should be adjusted with the control valve if necessary.

When the nebulising cycle is started, the compressed air flow meter should be at 10 CFH, and adjusted if required. The room containing the chamber was then vacated until the process ended (110 minutes), and the door appropriately marked to prevent intrusion. After completion of the cycle, the instrument was switched off, the lid opened, and the mice returned to their cages. All the contaminated components were wrapped up, sterilized by autoclaving, scrubbed clean and then autoclaved once more.

### 3.2.4 Determination of Initial Pulmonary Infection (Day 1)

To determine uptake of *Mycobacterium tuberculosis* into the lungs of the mice, 5 animals were sacrificed by cervical dislocation one day after aerogenic challenge. Entire lungs were removed and immediately placed into sterile tubes containing 2ml sterile saline solution. After completion of all necropsies, individual lungs were transferred into a sterile 10ml perspex mortar and homogenized with a pestle secured in a drill (Black and Decker KD574CRE, 680W). Lung homogenates were plated in duplicate volumes of 100µl and 200µl on Middlebrook 7H10 agar in duplicates, incubated at $37^0$C for 15-18 days, and CFU's enumerated.

### 3.2.5 TREATMENT OF INFECTED MICE

Treatment was started 14 days postinfection and mice divided into 5 per group. A control group of infected mice was sacrificed at the start of treatment (Early control group). A second group of infected but untreated mice was sacrificed two weeks after therapy was initiated (Late control group). Treatment was given once daily for 5 days by oral gavage except positive control (given in drinking water) for 2 weeks. Mice were injected with 0.2ml of the treatment solution using a 1ml syringe fitted with an atraumatic stainless steel oral dosing needle (Sterilin).

| SUNDAY | MONDAY | TUESDAY | WEDNESDAY | THURSDAY | FRIDAY | SATURDAY |
|---|---|---|---|---|---|---|
| Weigh mice | Aerosol infection | 1(Sacrifice) | 2 | 3 | 4 | 5 |
| 6 Weigh mice | 7 | 8 | 9 | 10 | 11 | 12 |
| 13 Weigh mice | 14(Start) Sacrifice | 15 | 16 | 17 | 18(Stop) | 19 |
| 20 Weigh mice | 21(Start) | 22 | 23 | 24 | 25(Stop) | 26(Sacrifice) |

Table 3.1. Experimental plan for treatment of tuberculosis.

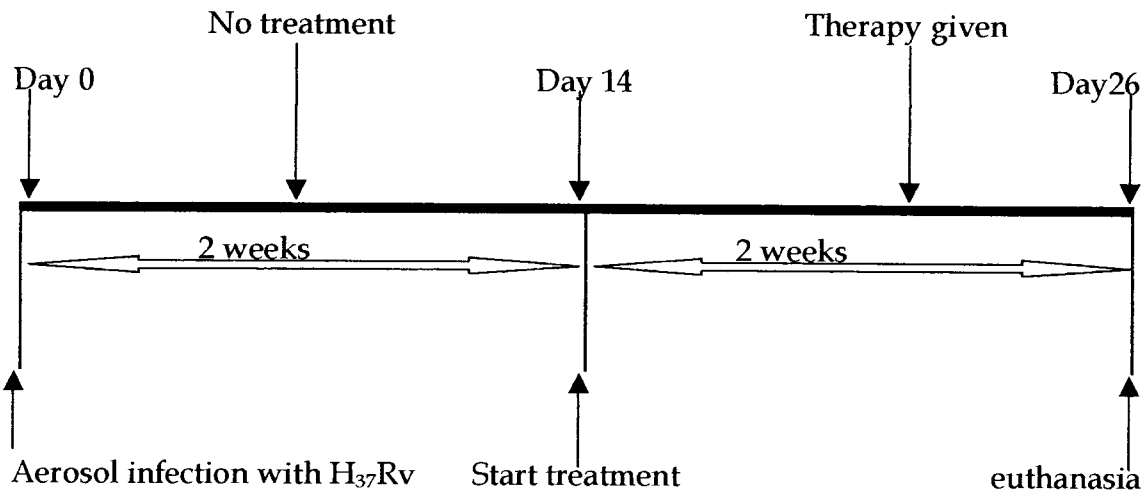



Fig 3.1. Schematic diagram of the experimental design for therapy of tuberculosis in mice.

### 3.2.6 POSITIVE CONTROL

A 1g of Rifampicin (Sigma Chemicals Co, South Africa) was dissolved in 10ml DMSO (Dimethyl Sulfoxide) solution and a 1g of Isoniazid (Sigma Chemicals Co, South Africa) was dissolved in 10ml of distilled water to make a 0.1g/ml stock of each. From each stock 200µl was removed and added to a bottle containing 200ml of drinking water to make the final concentration 0.1g/L. The bottle was changed weekly with fresh drugs.

### 3.2.7 TEST COMPOUND

For preparation of the test compound, crude plant extracts were weighed, dissolved in distilled water, and filter sterilized. Each animal received vehicle equivalent per kilogram of body weight (e.g. 100mg/kg). The plant extracts were freshly prepared each morning prior to administration.

### 3.2.8 CFU DETERMINATION IN ORGANS

A day after the last dose of treatment, treated and control mice were anaesthetized with diethyl ether before cervical dislocation. The lung, liver and spleen were aseptically removed (a piece cut for histology), weighed and immediately placed into sterile tubes containing 2ml of 0.9% Tween/Saline. After all necropsies were completed, individual organs were transferred into sterile 10ml perspex mortar and homogenized with a pestle secured in a drill (Black and Decker KD574CRE, 680W). Ten-fold serial dilutions of the homogenates was plated in duplicates on Middlebrook 7H10 agar plates, supplemented with 10%OADC and the plates incubated at 37$^{0}$C in $CO_2$ for 3 weeks before counting the CFU's.

### 3.2.9 TOXICITY DETERMINATION

Uninfected mice were treated with the same crude plant extracts used to treat the infected mice. Treatment was orally by gavages for a period of 2 weeks. Mice were observed for weight loss, ruffled fur, hunched posture, survival rate and other signs of distress. A record was kept of all mortalities and signs of toxicity. Lungs of mice that died were removed and tissue samples prepared for microscopical examination in an attempt to identify the cause of death. After treatment was stopped surviving mice were further observed for 5 days.

### 3.2.10 STATISTICAL ANALYSIS

All tests for significance were performed using the Student *t* test, by the Sigma plot program. *P* values of $< 0.05$ were considered significant. Means $\pm$ standard errors of the means were determined.

## 3.3 RESULTS

### 1. Establishment of aerosol infection model

An animal model was established to evaluate the activity of traditional herbs used in the treatment of tuberculosis in South Africa. Laboratory infection with *M. tuberculosis* was established by exposing mice to mycobacterium, aerosolised at 1 X $10^7$ cfu/ml. Day 1 sacrifice confirmed a delivery of between 100 - 130 cfu/lung in the infected mice (Fig 3.3A). The bacterial count in the lungs of infected mice increased by 2 log units between day 1 and day 14 ($p<0.05$). There was no significant difference in bacterial count ($p>0.05$) between early-untreated control (Day 14) and late-untreated controls (Day 26).

### 2. Inhibition of tuberculosis infection by INH-RIF combination

Treatment of infected mice was initiated on day 14 after infection. Treatment was once daily for 5 days per week and lasted for 2 weeks, then mice were sacrificed. INH-RIF combination, which is used widely in the early stages of the infection with *M. tuberculosis* was used as a positive control in the animal model for the evaluation of the activity of medicinal plant extracts against *M. tuberculosis*. Treatment of mice with INH-RIF resulted in the reduction of bacterial loads to below detection levels (Fig 3.3B).

### 3. Effect of herbal medicines

To be able to determine the optimum range of doses at which the potency of the anti-bacterial activity and toxicity of the medicinal plant extracts, could be evaluated. Mice were treated with plant extracts at different doses. It was found that some extracts could not dissolve at 1000mg/kg and this dose proved to be toxic in some cases. Subsequently 100mg/kg of body weight was chosen in the investigation of the anti-tuberculosis activity of plant extracts.

Treatment of mice with plant extract A, at different doses, did not inhibit the multiplication of the bacilli (Fig 3.4). Mice treated with plant extract A at 10, 100 and 500mg/kg of body weight respectively, produced between 1.5 - 2 log units increase in viable bacterial count in the lungs as compared ($p< 0.05$) to late-

untreated controls (LC). The increase in bacterial count suggested absence of antituberculous activity in plant extract A.

The anti-tuberculosis activity of plant extracts could in part involve inhibition of the dissemination of the bacilli in the body. Therefore, the spread of the bacilli from the lungs to other body organs after treatment with plant extracts was investigated. Plant extracts did not inhibit the spread of the bacilli to other body organs. Mice treated with 100mg/kg of other plant extracts were not efficacious in causing the bacterial elimination from the sites of infection (Fig 3.5). There was no significant reduction in bacterial counts in the liver and spleen of treated mice and the late-untreated controls.

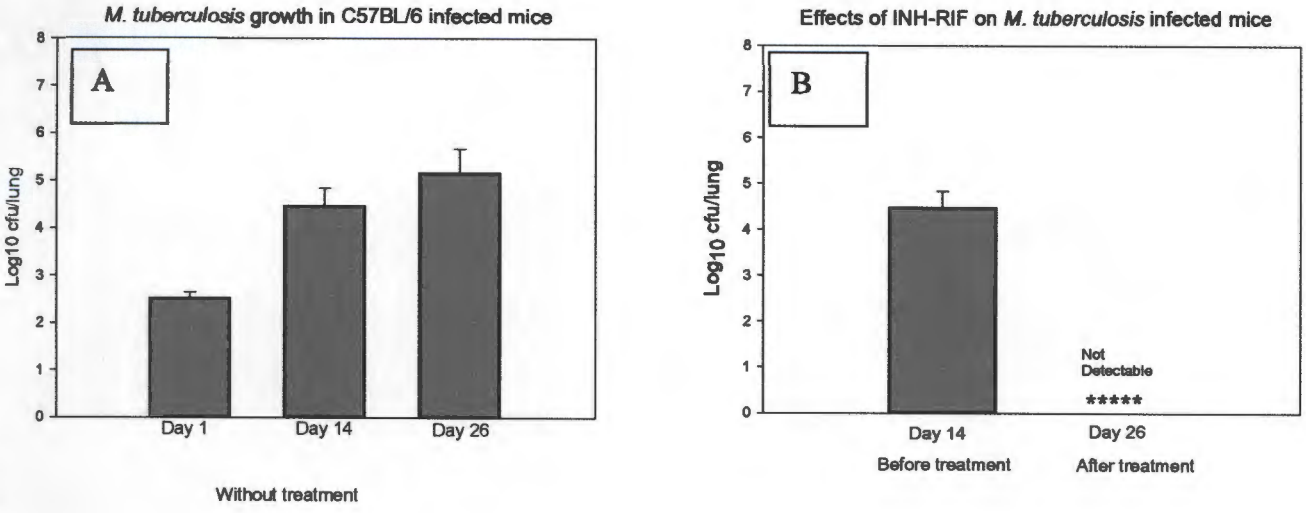



Fig 3.3 Number of viable *M. tuberculosis* organisms in lung of infected C57BL/6 mice (n=5). Lungs were aseptically removed, homogenized and plated out on agar plates for CFU counts. (A) Negative control – infected untreated mice sacrificed at different time points. (B) Mice treated with INH-RIF combination for 2 weeks. Results are means (solid bars) ± standard deviations (error bars).

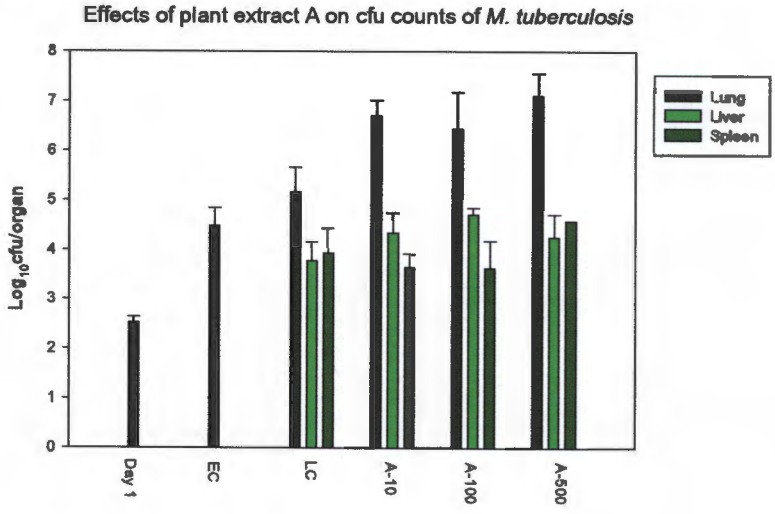



Fig 3.4 Number of viable *M. tuberculosis* organisms in lungs, livers and spleens of infected mice (n=5) after once-daily treatment for 2 weeks with the same plant extract at different doses. Organs were aseptically removed, homogenized and plated out on agar plates for CFU counts. Bacterial uptake confirmation mice were sacrificed at day 1. infected untreated mice from control groups were sacrificed at 2 weeks [early control (EC)] and at 4 weeks [late control (LC)] after infection. Results are means (solid bars) ± standard deviations (error bars)

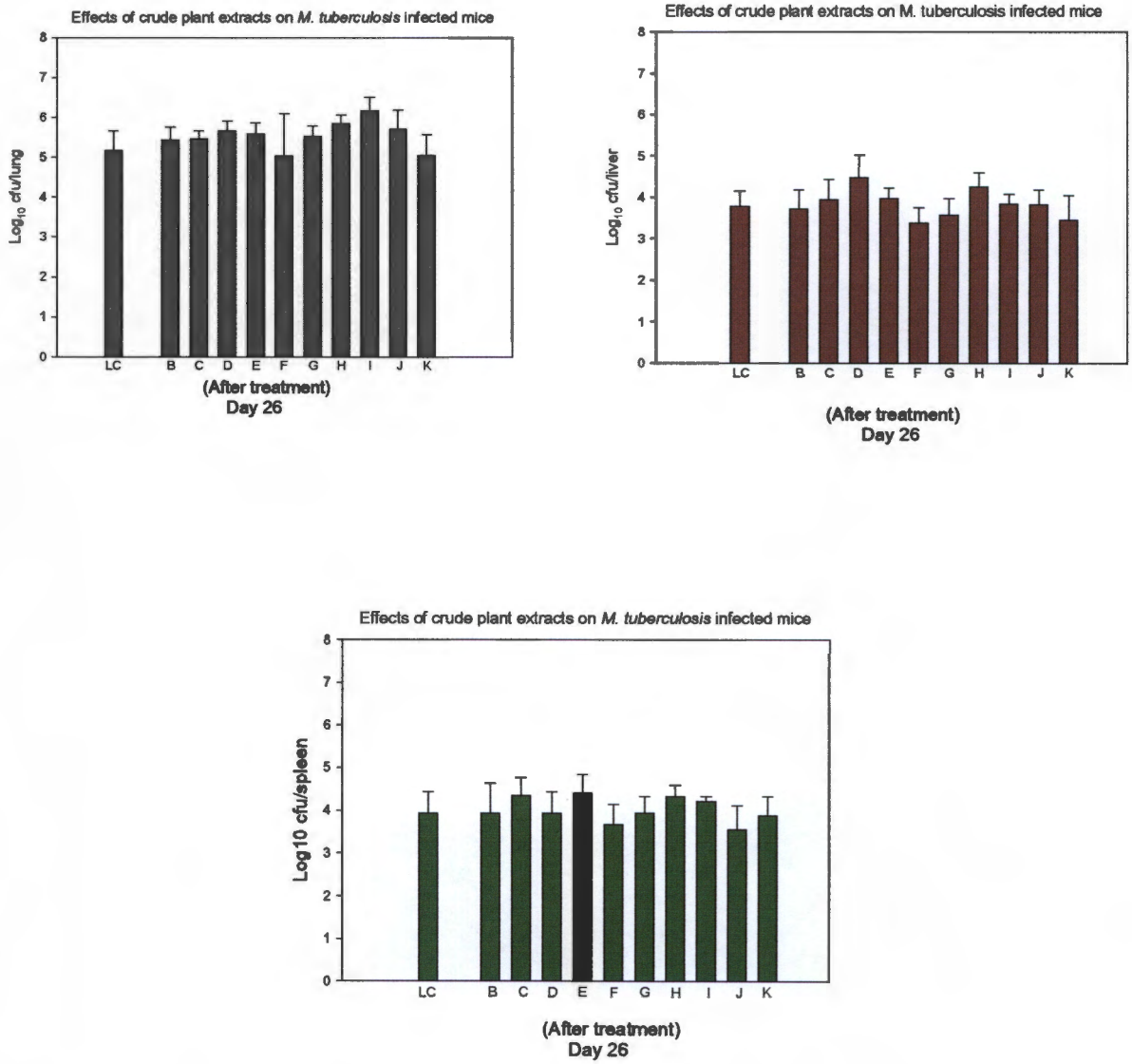



Fig 3.5. Number of viable *M. tuberculosis* organisms in lungs, livers and spleens of infected mice (n = 5) after once-daily treatment for 5 days per week for 2 weeks with different plant extracts at 100mg/kg of body weight. Organs were aseptically removed, homogenized and plated on the agar plates for CFU's. Infected untreated mice from control groups were sacrificed at 4 weeks (late control [LC]) after infection. Results are means (solid bars) ± standard deviations (error bars).

## 4. TOXIC EFFECTS OF PLANT EXTRACTS

To be able to determine the optimum range of doses at which the potency of anti-tuberculosis activity of the plant extracts could be investigated, a toxicity study on non-infected mice was simultaneously carried out and subsequently, a 100mg/kg of body weight was chosen for the investigation of anti-tuberculosis activity of plant extracts. Uninfected mice were given treatment for a period of 2 weeks by oral gavages. For each plant extract, 5 mice per group were observed for mortality, weight loss, ruffled hair and hunched posture.

As expected, before treatment was initiated the survival rate of the uninfected mice was 100%. Signs of reaction to treatment, observed shortly after dosing with crude plant extract B at 1000mg/kg, consisted of lethargy and pilo-erection. Mice in that group died 4 - 5 hours after the administration of the plant extract. They had hunched postures and ruffled hair on their death. Histology of the lungs revealed presence of blood in the alveolar spaces. Mice treated with the same extract at 100mg/kg resulted in 80% survival rate (**Fig 3.6**).

In most groups, survival rate was very high. In all groups where mice died, they started dying after treatment was initiated. Initiation of therapy resulted in minor loss of body weight during the first week and this normalized during the second week of treatment. No toxicity was observed in other groups during the course of therapy and plant extract administration was not associated with mortality. Survivors, as judged by external appearance and behavior, were apparently normal after 5 days of observation. This observation was substantiated by body weight gains (**Table 3.2**).

## Survival of C57BL/6 mice treated with crude plant extracts

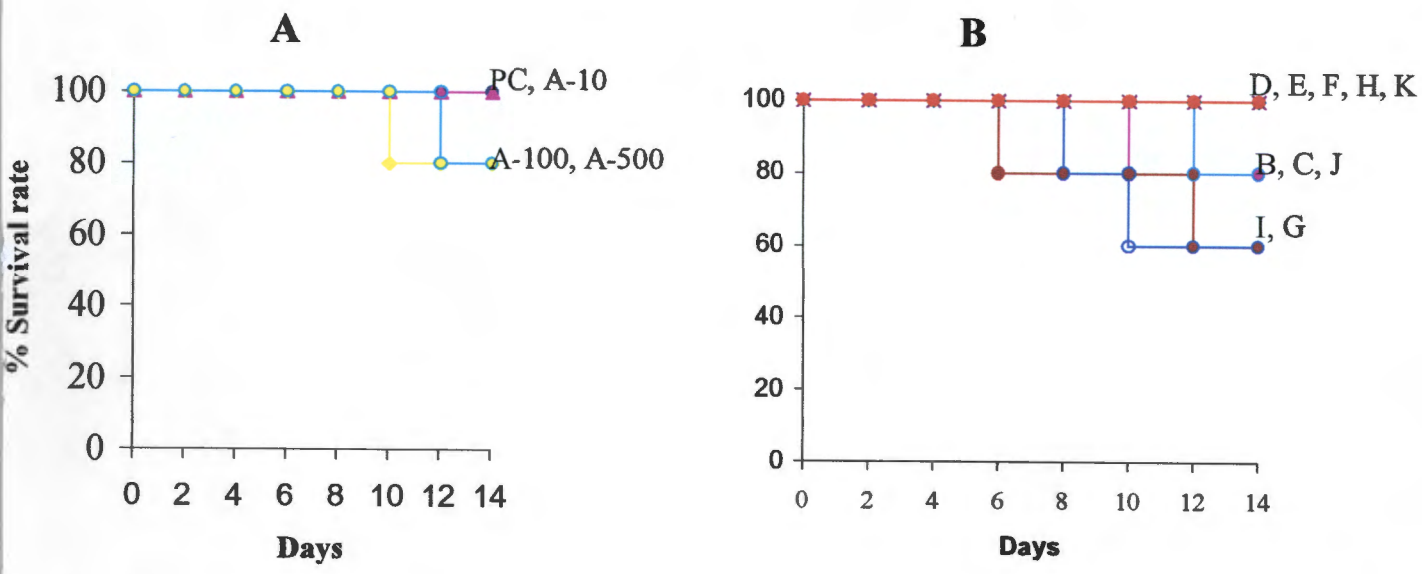



Fig 3.6. Effects of crude plant extracts on survival rates of uninfected C57BL/6 mice (n = 5). Mice were given treatment by oral gavages for 2 weeks. (A) Mice treated with plant extract A at different doses. (B) Mice treated with different plant extracts at same dose of 100mg/kg of body weight. PC- positive control given at 0.1g/L in drinking water.

**Table 3.2.** Group mean weight of mice dosed orally with different crude plant extracts

| Dose (mg/kg) | Body weight (g) at | | | |
|---|---|---|---|---|
| | Before treatment | Week 1 | Week 2 | 5 days after treatment |
| A- 10 | 20.46 | 19.62 | 21.60 | 22.36 |
| A- 100 | 20.62 | 20.26 | 22.31 | 22.35 |
| A- 500 | 25.52 | 23.28 | 24.45 | 25.52 |
| B- 100 | 26.27 | 22.87 | 25.67 | 26.27 |
| C- 100 | 17.34 | 16.90 | 17.59 | 19.33 |
| D- 100 | 20.77 | 19.06 | 20.88 | 20.91 |
| E- 100 | 19.44 | 18.35 | 19.46 | 19.02 |
| F- 100 | 26.02 | 24.79 | 24.63 | 26.02 |
| G- 100 | 26.38 | 25.26 | 25.42 | 26.38 |
| H- 100 | 25.68 | 24.93 | 24.75 | 25.68 |
| I- 100 | 24.32 | 23.77 | 24.80 | 24.32 |
| J- 100 | 26.71 | 25.29 | 25.91 | 26.71 |
| K- 100 | 23.53 | 23.44 | 24.80 | 26.78 |

## 3.4 DISCUSSION

Multidrug therapy is the cornerstone of any effective treatment regimen, and it prevents the emergence of drug resistance. Today, INH-RIF combination for the treatment of tuberculosis is still used to treat and prevent tuberculosis worldwide. Quenel et al (96) were able to completely eliminate CFU from the lungs of *M.tuberculosis* infected mice using a combination of microspheres loaded with Rifampin and oral Isoniazid after 26 days of treatment. In another study, Lenaerts et al (20), managed to achieve clearance in the lungs and spleen of infected mice after 12 weeks of oral INH-RIF treatment. In this study, we were able to completely eliminate CFU from the lung, liver and spleen using INH-RIF combination in drinking water after 2 weeks of treatment.

The most common usage of medicinal plants is as decoction or tea prepared by maceration with boiling water. This may in part be simply due to ease of preparation but also may reflect the involvement of water-soluble substances, however some carryover of less-polar substances could also be anticipated in crude preparations (18,19). In vivo assessment of crude medicinal plant extracts against *M. tuberculosis* is very difficult, and to establish the efficacy of these extracts, the animal model must closely resemble human infection. Gangadharan et al (46) have shown, at 4 weeks after infection, that the lungs of untreated infected mice exhibited histiocytic proliferation in the alveoli and alveolar septa, involving approximately 50% of the parenchymal area. Thus, the activities of medicinal plant extracts in these conditions might be different from those in an early infection. A chronic established infection, closer to the human infection, is more difficult to eradicate than an early acute infection.

We evaluated in this model of established infection of C57BL/6 mice with *M. tuberculosis* H37Rv strain the effects of crude medicinal plant extracts used by traditional doctors as a mixture of different plants or singly to treat tuberculosis. Traditional doctors combine known plants for treating tuberculosis because of their ability to act synergistically or because of treating different symptoms of the same disease.

Our studies have revealed that none of the tested medicinal plant extracts showed any activity against *M. tuberculosis*. This might be because the extracts were not being absorbed in the gastric system of the mouse or not enough active material was distributed to the sites of infection since these materials are likely to be in very low concentrations in these complex mixtures. Metabolic activity of mice is different from that of human and this might have been another possible contributing factor in our study. Our experiments also show that some plant extracts are toxic at very high concentrations and they lead to death. However, in some cases death was due to an artefact by gavage.

Testing medicinal plants for long-term treatment might show different results from our 2 weeks treatment model. It might also be important to start treatment immediately after infection to save time. Intravascular injection of mice with medicinal plants or giving test compounds in drinking water might also be important. Although the pathophysiology of tuberculosis in mice is different from that in humans, mice remain the best and most economical option for initial therapy evaluation (20). The discriminant power of this model is confirmed by the good activity of the positive control, INH-RIF combination, against established experimental infection.

# CHAPTER 4

# IMMUNOSTIMULATORY EFFECTS OF PLANT EXTRACTS ON CYTOKINE INDUCTION AND MICROSCOPIC ANALYSIS OF INFECTED ORGANS

## 4.1 INTRODUCTION

Among bacterial infections, mycobacterial diseases have the highest morbidity and mortality rates worldwide (21). This is primarily true for the causative agents of tuberculosis and leprosy, *M. tuberculosis* and *M. leprae*. Acquired cellular immunity to *M. tuberculosis* infection is characterized by the emergence of a population of protective CD4 T cells that secrete cytokines, resulting in local activation of macrophages and the recruitment of monocytes to initiate granuloma formation (22). The kinetics of this protective immunity, which leads to the control and containment of the infection, and the onset of bacterial clearance, is closely associated with the kinetics of emergence of CD4 T cells that secrete large amounts of the cytokine IFN-γ.

In vivo and in vitro infection with *M. tuberculosis* results in the secretion of numerous cytokines. Among the secreted cytokines TNF-α and IL-12 have powerful immunopotentiating effect. It also has the ability to initiate the development of Th1 phenotype in naïve T cells and the ability to potentiate IFN-γ production in antigen-activated Th1 cells.

*M. tuberculosis* persists in macrophages within a granuloma in the organs of infected hosts. The granuloma consists of macrophages and giant cell, T cells and fibroblasts. In this chapter, the type and presence of granuloma in plant treated mice was determined and the cytokine induction by plant extract was determined.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Bleeding of mice

Mice were anesthetized with diethyl ether. The sternum of the mouse was lifted and a 1ml syringe fitted with a 27,5 gauge needle (Sterilin) inserted under the sternum into the heart. Blood from the heart was collected and the plunger withdrawn gently. The collected blood was transferred into an eppendorf tube (Sterilin) and centrifuged at 14 000rpm for 10 minutes. Serum was then collected into a new eppendorf tube and stored at -70°C until use.

### 4.2.2 Cytokine measurements in serum

Serum was thawed before detection of cytokines by sandwich ELISA method (Enzyme Linked Immunosorbent Assay). The concentration of the cytokines IL-12 and IFN-γ was measured. Nunc-Maxisorp 96 well microtiter plates were coated with 100µl/well of the appropriate coating antibodies (biotin rat anti-mouse IFN-γ and IL-12p40), diluted in 'coating buffer' (0.1%BSA/PBS/.02% sodium azide[Merck]) and incubated at 4°C overnight. Plates were washed three times with PBS and incubated with 150µl blocking buffer (4% BSA/PBS/0.04% sodium azide) for 2 hours at 37°C to block residual binding sites on the plastic. Plates were next washed four times with washing buffer (0.05% Tween20 [Sigma]/PBS/0.01% sodium azide) and incubated with 50µl/well samples diluted 3-fold in dilution buffer and 50µl/well appropriate cytokine standards included in 12 three-fold dilutions. This was then incubated overnight at 4°C. After incubation the plates were washed again and incubated with 50µl/well of the capture antibody (see appendix) for 2 hours at 37°C. The plates were then washed and alkaline phosphatase labeled streptavidin (1/1000) added at 50µl/well and incubated for 1 hour at 37°C. The substrate P-Nitrophenol-Phosphate (Boehringer Mannheim) was diluted in substrate buffer 50µl was added to each well. The plates were read at 405/492 nm in a microplate

spectrophotometer (Molecular Devices, Spectra MAXGemini). To stop the reaction 50µl 1M sodium hydroxide was added.

### 4.2.3 Organ preparation for histology

Mice were sacrificed at week 2 and week 4 after infection. Lungs were aseptically removed from the week 2 mice. Lungs, liver and spleen were removed from the week 4 mice. Removed organ were weighed and a small portion of each tissue was placed in a solution of 10% formalin (4% formaldehyde in PBS pH 7.4). The organ sections were left in solution for 2 days to ensure killing of bacilli. Sections were then processed overnight and embedded in paraffin wax (Histosec Pastilles, Merck). Sections for Haematoxylin and eosin (H&E), as well as for Ziehl Neelsen (ZN) staining were cut to 4 µm thickness with a microtome (Leica, model RM-2125) and mounted on APES (3-aminopropyltriethoxysilane, 99%) coated slides.

### 4.2.4 Ziehl-Neelsen for Acid-fast Bacilli (AFB)

Sections were dewaxed overnight by incubating in an oven at 56$^0$C. Sections were placed on a staining rack and flooded with filtered Carbol Fuchsin, flamed until steaming and then left to cool for 5 minutes. The flaming-steaming process was repeated as before and followed by rinsing in water. Excess color was removed by rinsing with 1% acid alcohol (1% hydrochloric acid in 70% alcohol) for 30 seconds. The sections were washed in water before incubating in 25% $H_2SO_4$ for 20 minutes at room temperature. Slides were washed in running water for 10 minutes to remove acid and counterstaining was done in Loefflers' methylene blue for 1 minute at room temperature. Sections were washed in water, dehydrated through alcohol (70%, 70%, 90%, 96% and absolute alcohol) into Xylol for a few seconds and mounted in Entellan mounting medium (Merck).

### 4.2.5 Haematoxylin and Eosin for morphology

Sections were dewaxed overnight by incubating at 56$^0$C in an oven. The next day, sections were rehydrated by immersing them once in xylol for 3 minutes and twice in the same solution for 1 minute each. Sections were then washed twice in absolute alcohol for 1 minute each, twice in 96% alcohol for 1 minute each and once in 70% alcohol for 1 minute. Alcohol from sections was washed in running water for 1 minute. The sections were placed in a haemotoxylin solution for 8 minutes and then washed in water for 1-2 minutes. Excess staining was removed by immersion in 1% acid alcohol for 10 seconds, and the blue color retrieved by leaving sections in water for 30 minutes. Sections were counterstained in 1% eosin for 2 minutes and then washed in water. Sections were then dehydrated by passing them through 70%, 96% alcohol and finally through xylol. The sections were mounted with entellan and coverslipped.

### 1.2.6 Microscopic analysis of sections

The microscopic sections were investigated with light microscope and photographs were taken. Quantitative analysis of sections was done to determine tissue alterations.

## 4.3 RESULTS

### ACID FAST BACILLI (AFB)

Treated and untreated mice were examined for the presence of AFB in the lung, liver and spleen. Since plant extract A treated mice showed increased bacterial counts in the lungs, this increased growth of mycobacterium was visualized by the Ziehl-Neelsen staining. The lung sections of plant extract A treated mice at different doses contained abundant mycobacteria as compared to the untreated mice at week 4 (Fig 4.1). Untreated mice had few mycobacteria at 2 weeks as compared to 4 weeks. Increased mycobacterial growth of plant extract A treated mice was not dose dependant. INH-RIF treated mice did not show any mycobacteria in the lung, liver and spleen tissue sections.

### GRANULOMA FORMATION

Treated and untreated organs of *M. tuberculosis* infected mice were examined for granuloma formations with Haematoxylin and Eosin staining. Due to the inability of the plant extract to clear the bacilli, we investigated whether proper granulomas were formed; especially in plant extract A treated mice. This was done to determine the control of infection by plant extract A treated mice. Mice treated with plant extract A at different doses showed the presence of increased granulomas in the lungs. Lung tissues in treated group were compact and consisted of little air spaces. Mice treated with INH-RIF for 2 weeks showed infiltration with lymphocytes and macrophages in the lungs, but there was no granuloma formation. At 2 weeks after infection, the lungs of untreated mice showed no granuloma formation. Lung tissue was infiltrated with recruited macrophages and lymphocytes. Typical granulomas were found at 4 weeks post-infection in the lung of untreated mice.

## CYTOKINE LEVEL IN SERUM

We examined the expression of IL-12 in serum from treated and untreated mice infected with *M. tuberculosis*, because of its role in activating immune response and in engendering protective immunity by promoting the development of a Th1 T-cells. IL-12 is essential in control of *M. tuberculosis* infection. IL-12 levels were measured at 4 weeks postinfection by sandwich ELISA. This was done to determine if plant extracts had immunostimulatory effects on infected mice. There was no significant difference between positive (INH-RIF) and negative (untreated) control mice ($p < 0.005$) in the production of IL-12p40. IL-12p40 production was virtually suppressed in plant extract A treated mice, both at 100 and 500mg/kg.

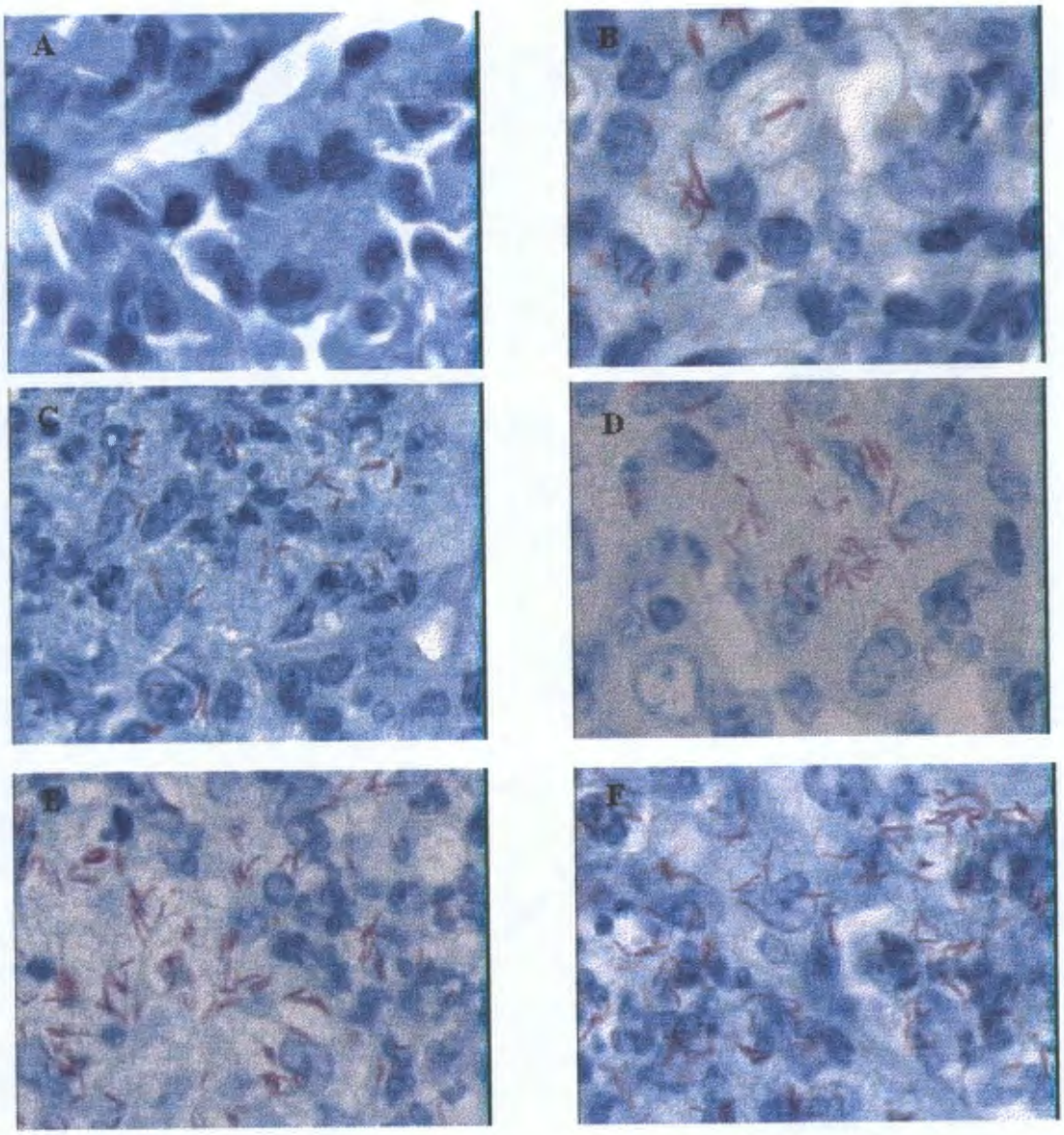



**Figure 4.1.** Histopathological studies (Ziehl-Neelsen stain) of lung tissues from treated and untreated C57BL/6 mice infected with *M. tuberculosis* (n = 5). Mice infected with 1 X $10^7$ CFU of H37Rv organisms. Infected mice were sacrificed, lungs asceptically removed and prepared for histological analysis. (A) INH-RIF treated mice – week 4. (B) Untreated mice – week 2. (C) Untreated mice – week 4. (D, E, F) Mice treated with different doses (10, 100, 500 mg/kg) of plant extract A, respectively – week 4. Original magnification X100.

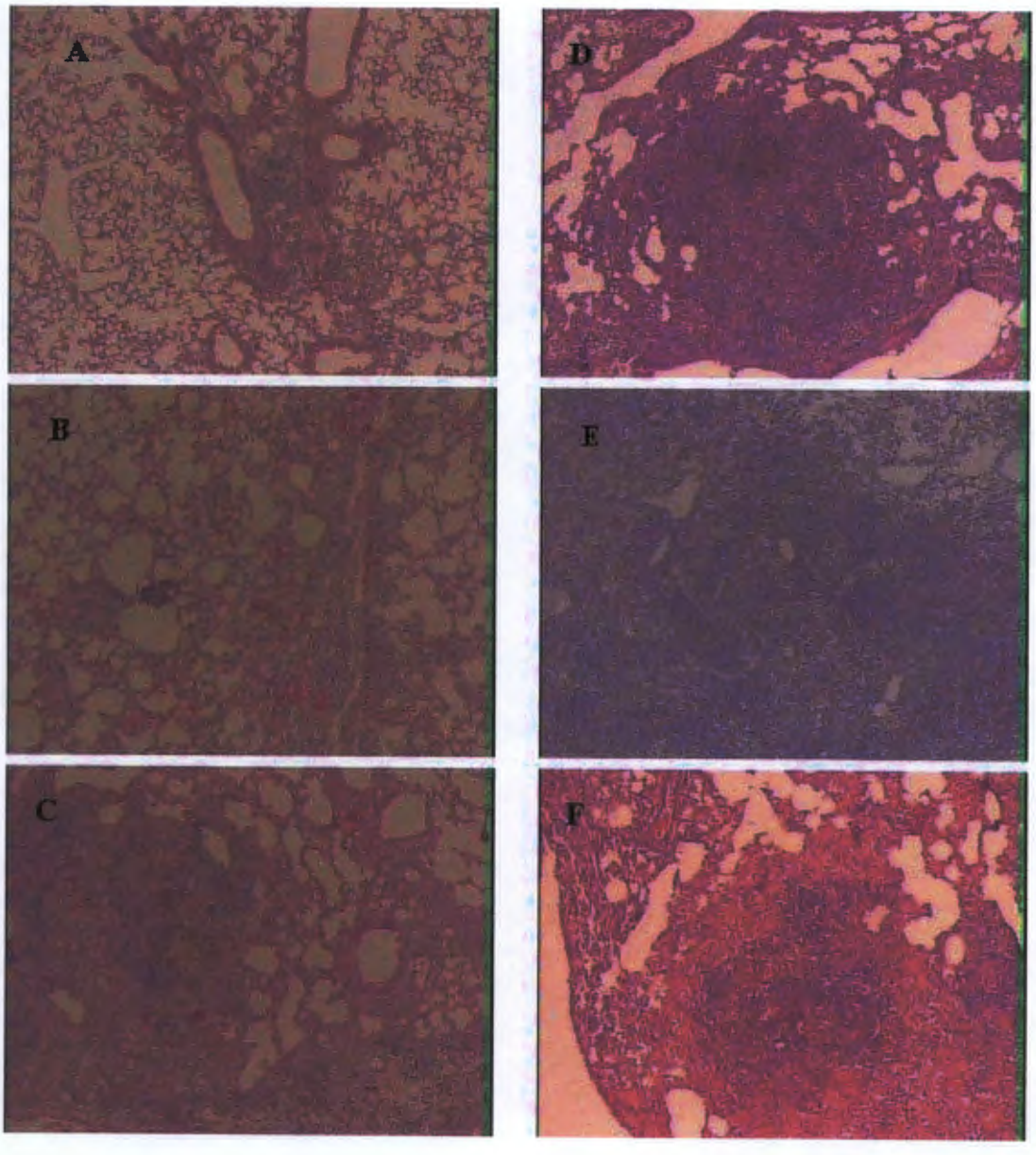



Fig 4.2. Histopathological studies (Haematoxylin and Eosin stain) of lung tissues from treated and untreated C57BL/6 mice infected with *M. tuberculosis*. Mice infected with 1 X $10^7$ CFU of H37Rv organisms (n = 5). Mice were sacrificed, lungs aseptically removed and prepared for histological analysis. (A) INH-RIF treated mice. (B) Untreated mice – week 2. (C) Untreated mice – week 4. (D, E, F) Mice treated with different doses (10, 100, 500 mg/kg) of plant extract A, respectively – week 4. Original magnification X4.

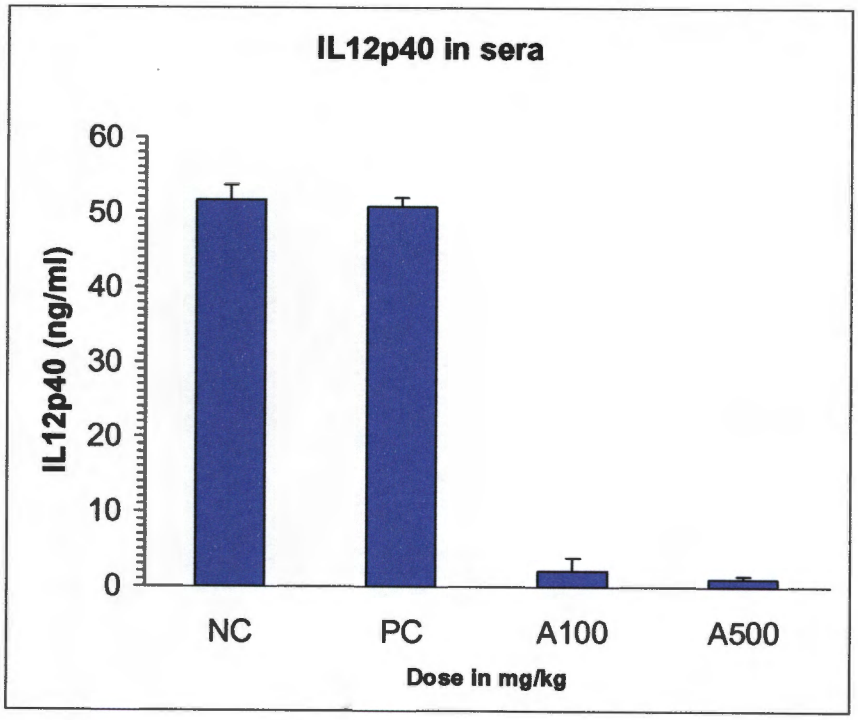



Fig 4.3. Effect of plant extract A on the production of IL-12p40 from serum of *M. tuberculosis* infected mice (n = 5). Infected mice were treated for 2 weeks with different doses of plant extract A. Serum samples were assayed by sandwich ELISA. Negative control (NC) – untreated mice. Positive control (PC) – INH-RIF treated mice. The data represents the means (solid bars) and ± standard deviations (error bars).

## 4.4 DISCUSSION

The immune response to all pathogens is at least in part dependent on cytokines, which regulate all cells of the immune system. *M. tuberculosis* is no exception and in fact strongly induces cytokines during infection. IL-12, which is induced during mycobacterial infection, is crucial in controlling *M. tuberculosis* infection (23). In our studies, we further evaluated the immunostimulatory effects of medicinal plant extracts on cytokine induction and the histology of infected organ.

Our mouse model of mycobacterial aerosol infection demonstrates that in the presence of IL-12, cells that are necessary for granuloma formation were recruited leading to a successful granuloma formation. INH-RIF treatment did not interfere with the production of IL-12 and the lung tissue was cleared of the bacteria indicating the healing activity of IHN-RIF combination.

IL-12 is one of the principal cytokines that drive the immune system to a Th-1 response, critical in the control of *M. tuberculosis* infection. Flynn et al (22) have shown that in the absence of IL-12, mice infected with *M. tuberculosis* were susceptible to infection and had a greatly increased bacterial burden. In another study, Cooper et al (23) have also shown that the absence of the bioactive IL-12 molecule results in unrestrained growth of *M. tuberculosis* bacteria in all target organs after infection. The absence of IL-12, also leads to the reduction in the level of IFN-$\gamma$ and the expression of TNF-$\alpha$ is delayed. The delay of IFN-$\gamma$ expression results in the delay of the induction of macrophage activation, which is mirrored by the lower number of lymphocytes in the lung granuloma. This leads to a formation of a defective granuloma. Therefore, the suppression of IL-12p40 production in *M. tuberculosis* infection after treatment with plant extract A was puzzling. The suppression of IL-12p40 in plant extract A treated mice did not demonstrate residual resistance to *M.tuberculosis* which is dependant on the

presence of endogenous IL-12p40. Thus rather than curtailing the infection, treatment with plant extract A may favor the survival and multiplication of *M. tuberculosis* in the host. This suppression might have been caused by production of other cytokines such as IL-4, IL-10 or TGFB. This reasoning would not contradict the increased bacterial burden observed in mice treated with plant extract A.

# CHAPTER 5

## OVERALL CONCLUSION

Tuberculosis is acquiring increasing importance throughout the world especially with the advent of Human Immunodeficiency Virus (HIV) infections. Together with HIV, it is the worst infectious disease facing the world. In South Africa, we are burdened by one of the worst tuberculosis epidemics ever recorded. The highest rates are recorded from the Western, Eastern and Northern Cape, where incidence figures (Chapter 1) are more than double those reported from the other provinces.

A once-off contact with traditional doctors when collecting information about the use of medicinal plants creates the impression that scientists exploit their knowledge and when activity is found they are not acknowledged for their contribution. This leads to a situation where traditional doctors end-up not trusting scientists and refusing to share information on the use of medicinal plants. Regular contacts should be maintained so that traditional doctors get feedback of the laboratory experiments done on medicinal plants and what they mean. Although the perception of tuberculosis between "Western" medicine and traditional medicine (Chapter 2) is different, traditional doctors should also be taught what western doctors know about tuberculosis (e.g. symptoms and cause)

A colleague, Eliya Madikane, also tested the plant extracts used in this study *in vitro* by the Luciferase Reporter Mycobacteriophage assay (LRP). He tested organic extracts of the same plants against *Mycobacterium aurum*. His results show activity by plant extract I. His preliminary results of fractions from this extract tested against H37Rv also show some activity. In our study, water was used, as the solvent for extraction, there is the possibility that the active

used, as the solvent for extraction, there is the possibility that the active ingredients could have been insoluble in water and as such not extracted in sufficient amounts.

We could not identify any activity in the in vivo aerosol H37Rv infection studies (Chapter 3). This does not mean that these medicinal plants are not effective at all against other chest diseases. Although these plants are reported as being used to treat tuberculosis or symptoms similar to that caused by it, it is possible that these plants are effective against colds, coughs or chest pains caused by diseases other than tuberculosis. Laboratory tests to confirm tuberculosis in patients treated by traditional doctors should be done before and after treatment with medicinal plants so that efficacy of treatment can be identified. It is well established that most drugs have adverse effects. However, in contrast to herbal products, conventional drugs undergo trials prior to registration by the Medicines Control Council (MCC), followed by post-marketing surveillance that documents adverse effects. We found that plant extract B was toxic at high concentrations (1000mg/kg) and traditional doctors must be informed to avoid complications associated with the prescription of that plant. This should be seen as our moral obligation to protect the health and safety of our people with respect to use of herbal medicines.

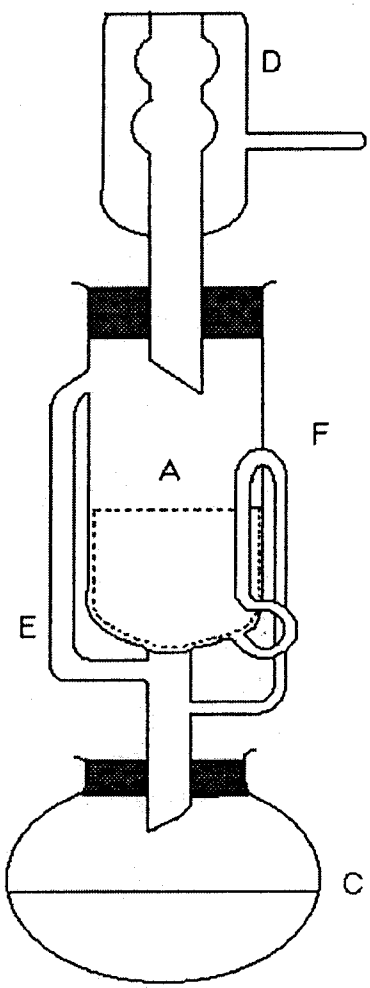
Plant extract A suppressed the immune production of IL-12 (Chapter 4) in infected mice. This extract might be useful in treating Th1 mediated autoimmune pathologies caused by the overproduction of IL-12 (e.g. systemic sclerosis). Ethanol has been shown to up-regulate IL-12 production in human monocytes and indeed, increased serum IL-12 levels have been associated with chronic alcoholism (73). It is therefore possible that the effect of ethanol on IL-12 can be counteracted by the use of plant extract A, but as to whether plant extract A also suppresses IL-12 production in humans remains to be established.

The mouse model has been used to test the antituberculous activity of several drugs because it is highly reproducible and inexpensive. The course of the disease that follows experimental infection with *Mycobacterium tuberculosis* is different from that of the disease in humans. However, the mouse model is able to reproduce bacillary populations of comparable size to those observed in the lung cavity of human tuberculosis (38). Therefore the mouse tuberculosis model can be safely used for discovering prototypes of new compounds with new activities against *Mycobacterium tuberculosis*.

# APPENDIX A

# SOXHLET APPARATUS FOR PLANT EXTRACTION

**Appendix A**




**Principle of the Soxhlet Extraction:** The plant material is placed in **A**, the Soxhlet apparatus. The apparatus is then fitted onto flask **C**, which contains the solvent. The solvent is boiled gently. Vapour passes up through the tube **E** which is condensed by the condenser **D**. The condensed solvent falls into **A** and slowly fills the body of the Soxhlet. When the solvent reaches the top of tube **F**, it siphons over into flask **C** and thus removes that portion of the substance which it has extracted in **A**. This process is repeated automatically until the extraction is complete, usually over several hours. The extracted compound is then isolated from **C** by evaporating the solvent (84).

# APPENDIX B

# EXPERIMENTAL REAGENTS

# APPENDIX B

## A. BACTERIOLOGICAL REAGENTS

### A.1. Difco Middlebrook 7H10 Agar (M7H10)

19g M7H10 agar and

5ml glycerol were made up to 900ml with

distilled water ($H_2O$) and autoclaved at 121$^0$C for 10 minutes.

The solution was allowed to cool to 55$^0$C and 100ml of oleic acid albumin catalase (OADC) or albumin dextrose catalase (ADC) added. Seven millilitres of the agar solution was poured per side in sterile duplicate petridishes (Sterilin). When set, the plates were stored inverted at 4$^0$C. the agar was brought to room temperature 1 or 2 hours before use.

### A.2. Albumin Dextrose Catalase (ADC)

25g Bovine Serum Albumin (BSA)

10g Glucose D, and

4.25g Sodium Chloride (NaCl) were dissolved in

500ml distilled $H_2O$

the solution was then filtered first through a 0.45μm millipore filter. Hundred millilitre aliquots were made and stored at 4$^0$C.

### A.3. 0.9% Saline solution

9g of NaCl was dissolved in

100ml of distilled $H_2O$ and then autoclaved at 121$^0$C for 30 minutes. The solution was stored at room temperature.

### A.4. Homogenizing Buffer

0.9% NaCl

0.04% Tween 80

dissolved in distilled $H_2O$.

Autoclaved for 30 minutes at 121$^0$C and stored at room temperature.

## B. GENERAL REAGENTS

### B.1. 10 X Phosphate Buffered Saline (PBS)

80g NaCl (1.37M)

2.4g $KH_2PO_4$

2g KCl (0.03M) and

14.4g $Na_2HPO_4.2H_2O$ were dissolved in

900ml distilled $H_2O$

The pH was adjusted to 7.4 and made up to 1000ml with distilled $H_2O$. The solution was sterilized through a 0.45μm millipore filter and stored at room temperature. The buffer was diluted 1/10 as a working solution.

## C. ELISA REAGENTS

Bovine serum albumin (BSA) Fraction V Cat # 735 086 Boehringer Mannheim

4-Nitrophenylphosphat (PNPP) Cat # 738 352 Boehringer Mannheim

Diethanolamine Cat # Art 803116 Merck

Instant milk powder (Spar Supermarket)

Streptavidin-Alkaline phosphatase Cat # 13043E Pharmingen

### C.1. Coating Buffer

(0.02%) 0.2g $NaN_3$ was dissolved in

1000ml of 1 X PBS (pH 7.4)

The solution was sterilized through a 0.45μm millipore filter and stored at $4^0$C.

### C.2. Dilutin Buffer

10g (1%) BSA and

0.2g (0.02%) $NaN_3$ were dissolved in

1 X PBS (pH 7.4) and made up to 1 litre, sterile filtered through a 0.45μm millipore filter and stored at $4^0$C.

**C.3. Blocking Buffer**

20g (2%) Milk powder (Instant milk powder from Spar) or 40g (4%) BSA and 0.2g $NaN_3$ (0.02%) was added to

100ml 10 X PBS and made up to 1L with distilled $H_2O$.

The solution was stirred until properly dissolved, filterd through Whatmann filter paper no 1 and sored at $4^0$C. For BSA, the solution was filtered through a 0.45μm millipore filter.

**C.4. 20 X Washing Buffer**

20g KCl,

20g $KH_2PO_4$,

144g $Na_2HPO_4.2H_2O$,

800g NaCl,

50ML NaCl,

50ML Tween 20, and

100ml 10% $NaN_3$ were dissolved in

5L of distilled H2O.

The solution was filter sterilized through a 0.45μm millipore filter and stored at $4^0$C. The buffer was diluted 1/20 as a working solution.

**C.5. Substrate Buffer**

0.3g $NaN_3$,

97ml Di-ethanolamine (liquefy in $37^0$C waterbath), and

0.8 $MgCl_2.6H_2O$ were dissolved in

700ml distilled $H_2O$.

The pH was adjusted to pH 9.8 with

10M HCl and then made up to 1L. Filter sterilized through a 0.45μm millipore filter and stored at $4^0$C.

## D. HISTOLOGY REAGENTS

### D.1. Coating slides with APES (3-aminopropyltriethoxysilane, 99%)

Slides were washed in 1% Teepol diluted in distilled $H_2O$ for 10 minutes at room temperature shaking. Slides were then washed in running tap $H_2O$ for 1 hour and dried at 60$^0$C for 4 hours or overnight at 37$^0$C. Dried slides were then immersed 10 X in 3.3% APES in distilled $H_2O$. Slides were then dried overnight at 37$^0$C, packed in boxes and stored at room temperature for later use.

### D.2. Mayers Haematoxylin

Dissolve each of the following compounds in order ensuring that each one has dissolved properly before adding next one.

1g Haematoxylin

50g Ammonium alum (Aluminium ammonium sulphate) or Potassium alum (Aluminium potassium sulphate)

0.2g Sodium iodate

1g Citric acid

50g Chloral hydrate

Filter through Whatmann paper no 1 and store in dark place at room temperature.

### D.3. Eosin

150ml 1% Eosin in distilled $H_2O$ (2parts)

75ml 1% Phloxine in distilled $H_2O$ (1part)

225ml distilled $H_2O$

One drop formalin or thymol was added to the 1% eosin for preservation. Filter through Whatmann filter paper no 1 and store at room temperature.

**D.4. Carbol Fuchsin**

Stock: 6% Basic Fuchsin in absolute alcohol

Staining solution : 10ml stock

90ml 5% carbolic acid (phenol) in distilled $H_2O$.

Filter the solution through Whatmann filter paper no 1 and store the solution at room temperature.

**D.5. Buffered Formalin (fixative for tissues)**

10ml formaldehyde (40% w/v formaldehyde solution)

900ml PBS (pH 7.4)

store at room temperature in a dark bottle.

**D.6. Loeffers' Methylene Blue**

Stock: 0.8% Methylene blue in absolute alcohol

Staining solution: 30ml stock

99ml distilled $H_2O$

1ml 1% KOH

Filter the solution through Whatmann filter paper no 1 and store at room temperature.

**D.7. Schiffs (PAS) Reagent**

Dissolve 1g Basic Fuchsin in 200ml boiling distilled $H_2O$ in stopped 1L flask.

Shake for 5 minutes.

Cool to $50^0C$, filter , add20ml 1N HCl to filtrate.

Store 18-24 hours in dark.

Add 2g activated charcoal - shake 1 minute.

Filter - store at $0\text{-}4^0C$ in dark.

## REFERENCE


1. Rom WN, Garay S. 1996. Tuberculosis. Little, Brown and Company, Inc. New York.

2. Orme I.1995. Immunity to Mycobacteria. R.Glandes Company. Austin. 27-30.

3. Young DB, Duncan K. 1995. Prospects for new interventions in the treatment and prevention of mycobacterial disease. Annual reviews. Microbiology. 49: 641 - 673.

4. Bloom BR. 1994. Tuberculosis: Pathogenesis, Protection, and Control. American Society for Microbiology. Washington. 113-130.

5. Fourie B, Weyer K. 2000. TB and HIV - the deadly duo. MRC News. 31: 21-22.

6. Dannenberg AM. 1989. Immune Mechanisms in the Pathogenesis of Pulmonary Tuberculosis. Reviews of infectious diseases. 11: S369-S377.

7. McDonough KA, Kress Y, Bloom BR. 1993. Pathogenesis of Tuberculosis: Interaction of M.tuberculosis with Macrophages. Infection and Immunity. 61: 2763-2773.

8. Beyers N. 1999. Tuberculosis. Wellcome Trust Workshop on immunology. Dept.Immunology UCT, Cape Town.

9. Miyazaki E, Chaisson RE, Bishai WR. 1999. Analysis of Rifapentine for Preventive Therapy in the Cornell Mouse model of Latent Tuberculosis. Antimicrobial Agents and chemotherapy. 43: 2126-2130.

10. Bartmann K. 1988. Anti-tuberculosis Drugs. Springer-Verlag Berlin Heidelberg. Germany.

11. Reichman LB, Hershfield ES. 2000. Tuberculosis, A compehensive international approach. Marcel Dekker, Inc. New York.

12. AFHS Drug Information. 2000. American Society of Health-System Pharmacists. 485 - 506.

13. Katz SH, Kimani VN. 1982. Why patients go to the traditional healers. East African Medical Journal. 59: 170 - 174.

14. Coovadia HM, Benatar SR. 1991. A century of tuberculosis: South African Perspectives. Oxford University Press. Cape Town.

15. Fourie TG, Swart I, Snyckers FO. 1992. Folk medicine:a variable starting point for pharmaceutical research. South African journal of Science. 88: 190 - 192.

16. Cantrell CL, Nunez IS, Castaneda-Acosta J. 1998. Antimycobacterial activities of dehydrocostus lactone and its oxidation products. J. of natural products. 61: 1181 - 1186.

17. Konig GM, Wright AD, Franzblau SG. 1999. Assessment of antimycobacterial activity of a series of mainly marine derived natural products. Planta Medica. 66: 337 - 342.

18. Irobi ON, Moo-Young M, Anderson WA, Daramola SO. 1994. Antimicrobial activity of bark extracts of *Bridelia ferruginea.* J. Ethnopharmacology. 43: 185 - 190.

19. Panthong A, Kanjanapothi D, Taylor WC. 1986. Ethnobotanical review of medicinal plants from Thai traditional books, Part 1: Plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. J. Ethnopharmacology. 18: 213 - 228.

20. Lenaerts MJA, Chase SE, Chmielewski AJ, Cynamon MH. 1999. Evaluation of Rifapentine in long-term treatment regimens for tuberculosis in mice. Antimicrobial Agents and chemotherapy. 43: 2356 - 2360.

21. Ehlers S, Richter E. 2001. Differential requirement for interferon-γ to restrict the growth of or eliminate some recently identified species of nontubercululous mycobacteria *in vivo*. Clinical Experimental Immunology. 124: 229 - 238.

22. Flynn JL, Chan J. 2001. Immunology of tuberculosis. Annual reviews of Immunology. 19: 93 - 129.

23. Cooper AM, Magram J, Ferrante J. 1997. Interleukin 12 (IL-2) is crucial to the development of protective immunity in mice intravenously infected with *Mycobacterium tuberculosis*. J. Experimental Medicine. 186: 39 - 45.

24. Adams JFA, Scholvinck EH, Gie RP. 1999. Decline in total serum IgE after treatment for tuberculosis. The Lancet. 353: 2030 - 2032.

25. Alba RM, Souza B. 1996. How to study the pharmacology of medicinal plants in underdeveloped countries. J. Ethnopharmacology. 54: 131 - 138.

26. Albino JA, Reichman LB. 1998. The treatment of Tuberculosis. Respiration. 65: 237-255.

26. Albino JA, Reichman LB. 1998. The treatment of Tuberculosis. Respiration. 65: 237-255.

27. Bajolet O, Beguinot I, Brasme L. 2000. Isolation of an unusual Mycobacterium species from an AIDS patient with acute lymphadenitis. J. of Clinical microbiology. 38: 2018 - 2020.

28. Bekker L, Moreira AL, Bergtold A. 2000. Immunopathologic effects of tumor necrosis factor alpha in murine mycobacterial infection are dose dependent. Infection and Immunity. 68: 6954 - 6961.

29. Bermudez LE, Kolonoski P, Wu M, Aralar PA, Inderlied CB, Young LS. 1999. Mefloquine is active in vitro and in vivo against *Mycobacterium avium* complex. Antimicrobial Agents and chemotherapy. 43: 1870 - 1874.

30. Bhat RB, Jacobs TV. 1995. Traditional herbal medicine in Transkei. J. Ethnopharmacology. 48: 7 - 12.

31. Blom B. 1995. Holism, Definition and Principles. International Journal of alternative and complementary medicine. 14 - 17.

32. Brooks VJ, Orme IM. 1998. Evaluation of once weekly therapy for tuberculosis using Isoniazid plus Rifamycins in the mouse aerosol infection model. Antimicrobial Agents and chemotherapy. 42: 3047-3048.

33. Burgos RA, Caballero EE. 1997. Testicular toxicity assessment of *Andrographis paniculata* dried extracts in rats. J. Ethnopharmacology. 58: 219-224.

34. Bussing A, Schweizer K. 1998. Effects of a phytopreparation from *helleborus niger* on immunocompetent cells in vitro. J. Ethnopharmacology. 59: 139 - 146.

35. Cantrell CL, Rajab MS, Franzblau SG. 1999. Antimycobacterial Triterpenes from *Melia volkensii*. J. Natural Products. 62: 546 - 548.

36. Chang ZG, Kennedy DT, Holdford DA. 2000. Pharmacists' knowledge and attitudes toward herbal medicine. The Annals of Pharmacotherapy. 34: 710 - 715.

37. Cobelens FGJ, van Deutekom H. 2000. Risk of infection with *Mycobacterium tuberculosis* in travellers to areas of high tuberculosis endemicity. The Lancet. 356: 461 - 465.

38. Cohen Y, Perronne C, Lazard T, Truffot-Pernot C, Grosset J, Vilde J, Pocidalo J. 1995. Use of normal C57BL/6 mice with established Mycobacterium avium infections as an alternative model for evaluation of antibiotic activity. Antimicrobial Agents and chemotherapy. 39: 735 - 738.

39. Cooper AM, Flynn JL. 1995. The protective immune response to *Mycobacterium tuberculosis*. Current Opinion in Immunology. 7: 512 - 516.

40. Cynamon MH, Zhang Y, Harpster T. 1999. High-dose Isoniazid therapy for Isoniazid-Resistant murine *Mycobacterium tuberculosis* infection. Antimicrobial Agents and chemotherapy. 43: 2922 - 2924.

41. Daniel N, Lounis N, Ji B. 2000. Antituberculosis activity of Once-weekly Rifapentine-containing regimens in mice. American Journal of respiratory and critical care medicine. 161: 1572 - 1577.

42. Davis L, Kuttan G. 1998. Suppressive effect of cyclophosphamide-induced toxicity by *Withania somnifera* extract in mice. J. Ethnopharmacology. 62: 209 - 214.

43. Dobos KM, Spotts EA, Quinn FD. 2000. Necrosis of lung epithelial cells during infection with *Mycobacterium tuberculosis* is preceded by cell permeation. Antimicrobial Agents and chemotherapy. 68: 6300 - 6310.

44. Doherty TM, Sher A. 1998. IL-12 promotes drug-induced clearance of *Mycobacterium avium* infection in mice. J. of Immunology. 160: 5428 - 5438.

45. Furney SK, Skinner PS, Farrer J, Orme IM. 1995. Activities of Rifabutin,, Clarithromycin, and ethambutol against two virulent strains of *Mycobacterium avium* in a mouse model. Antimicrobial Agents and chemotherapy. 39: 786 - 789.

46. Gangadharam RJP, Ashterar DR, Flasher DL, Duzgunes N. 1995. Therapy of *Mycobacterium avium* complex infections in beige mice with Streptomycin encapsulated in sterically stabilized liposomes. Antimicrobial Agents and chemotherapy. 39: 725 - 730.

47. Gebhardt R. 2000. In vitro screening of plant extracts and phytopharmaceuticals: Novel approaches for the elucidation of active compounds and their mechanisms. Planta Medica. 66: 99 - 105.

48. Gessler MC, Msuya DE, Nkunya MHH. 1995. Traditinal healers in Tanzania: sociocultural profile and three short portraits. J. Ethnopharmacology. 48: 145 - 160.

49. Hamburger M, Hostettmann K. 1991. Bioactivity in plants: The link between phytochemistry and medicine. Phytochemistry. 30: 3864 - 3874.

50. Hardman JG, Limbird LE. 1995. Goodman & Gilman's The pharmacological basis of therapeutics. 9: 1159-1160.

51. Hirata T, Saito H, Tomioka H, Sato K, Jidoi J, Hosoe K, Hidaka T. 1995. In vitro and in vivo activities of the Benzoxazinorifamycin KRM-1648 against *Mycobacterium tuberculosis.* Antimicrobial Agents and chemotherapy. 39: 2295 - 2303.

52. Houghton PJ, Woldemariam TZ, Watanabe Y. 1999. Actvity against mycobacterium tuberculosis of Alkaloid constituents of Agostura bark, Galipea officinalis. Planta medica. 65: 250 - 254.

53. Hsu HY, Lin CC, Chen JY. 1998. Toxic effects of *Erycibe obtusifolia*, a Chinese medicinal herb, in mice. J. Ethnopharmacology. 62: 101 - 105.

54. Hurtig AK, Pande SB, Baral SC. 2000. Anti-tuberculosis treatment in private pharmacies, Kathmandu Valley, Nepal. International Journal of Turbecle and Lung Disease. 4: 730 - 736.

55. Imperiali FG, Zaninoni A, Maestra LA. 2001. Increased *Mycobacterium tuberculosis* growth in HIV-1-infected human macrophages: role of tumour necrosis factor-α. Clinical and Experimental Immunology. 123: 435 - 442.

56. Jacobs M, Brown N, Allie N. 2000. Fatal *mycobacterium bovis* BCG infection in TNF-LT-α-deficient mice. Clinical Immunology. 94: 192 - 199.

57. Jacobs M, Brown N, Allie N. 2000. Increased resistance to mycobacterial infection in the absence of interleukin-10. Immunology. 100: 949 - 501.

58. Jacobs M, Brown N, Allie N. 2000. Tumor Necrosis Factor 2 plays a minor role for Mycobacterial immunity. Pathobiology. 68: 68 - 75.

59. Jacobs M, Marino MW, Brown N. 2000. Correction of defective host response to *Mycobacterium bovis* BCG infection in TNF-defective mice by bone marrow transplantation. Laboratory Investigation. 80: 901 - 914.

60. Jagannath C, Allaudeen HS, Hunter RL. 1995. Activities of Poloxamer CRL8131 against *Mycobcterium tuberculosis in vitro* and *in vivo.* Antimicrobial Agents and chemotherapy. 39: 1349 - 1354.

61. Jagannath C, Emanuele MR, Hunter RL. 1999. Activities of Poloxamer CRL-1072 against *Mycobacterium avium* in macrophage culture and in mice. Antimicrobial Agents and chemotherapy. 43: 2898 - 2903.

62. Ji B, Lounis N, Maslo C. 1998. In vitro and In vivo activities of Moxifloxacin and Clinafloxacin against M. tuberculosis. Antimicrobial Agents and chemotherapy. 42: 2066-2069.

63. Ji B, Lounis N, Truffot-Pernot C, Grosset J. 1995. In vitro and in vivo activities of Levofloxacin against *mycobacterium tuberculosis.* Antimicrobial Agents and chemotherapy. 39: 1341 - 1344.

64. Kelly BP, Furney SK, Orme IM. 1996. Low-Dose Aerosol Infection model for testing drugs for Efficacy against Mycobacterium tuberculosis. Antimicrobial Agents and chemotherapy. 40: 2809-2812.

65. Kempsell KE, Cox CJ, McColm AA. 2001. Detection of *mycobacterium tuberculosis* group organisms in human and mouse joint tissue by reverse transcriptase PCR: Prevalence in diseased synovial tissue suggests lack of specific association with Rheumatiod Arthritis. Infection and Immunity. 69: 1821 - 1831.

66. Keplinger K, Laus G, Wurm M. 1999. *Uncaria tomentosa* (wild) DC.- Ethnomedicinal use and new pharmacological, toxicological and botanical results. J. Ethnopharmacology. 64: 23 - 34.

67. Lee EH, Kim NK, Hwang CY. 1998. Activation of inducible nitric oxide synthase by Yongdam-Sagan-Tang in mouse peritoneal macrophages. J. Ethnopharmacology. 60: 61 - 69.

68. Lenaerts AM, Chase SE, Cynamon MH. 2000. Evaluation of rifalazil in a combination treatment regimen as an alternative to Isoniazid-rifampin therapy in a mouse tuberculosis model. Antimicrobial Agents and chemotherapy. 44: 3167 - 3168.

69. Lipp FJ. 1989. Methods for ethnopharmalogical field work. J. Ethnopharmacology. 25: 139 - 150.

70. Lounis N, Bentoucha A, Truffort-Pernot C. 2001. Effectiveness of once-weekly Rifapentine and Moxifloxacin regimens against *Mycobacterium tuberculosis* in mice. Antimicrobial Agents and chemotherapy. 45: 3482 - 3486.

71. Lowrie DB, Tascon RE, Bonato VLD. 1999. Therapy of tuberculosis in mice by DNA vaccination. Nature. 400: 269 - 271.

72. Mandell GL, Dolin R, Bennet JE. 1995. Principles and Practice of Infectious Diseases. Fouth Edition. Churchill Livingstone. 2213-2231.

73. Marriott JB, Clarke IA, Dalgleish AG. 2001. Inhibition of p38 MAP kinase during cellular activation results in IFN-γ-dependent augmentation of IL-12 production by human monocytes/macrophages. Clinical Experimental Immunology. 125: 64 - 70.

74. Miller L. 1998. Herbal medicinals: Selected clinical considerations focussing on known or potential Drug-Herb interactions. Arch Intern Med. 158: 2200 - 2211.

75. Mohan VP, Scanga CA, Yu K. 2001. Effects of tumor necrosis factor alpha on host immune response in chronic persistent tuberculosis: Possible role for limiting pathology. Infection and Immunity. 69: 1847 - 1855.

76. Murray L, Chavunduka GL. 1986. The professionalisation of African medicine. Manchester University Press. Manchester.

77. Nilsson LE, Hoffner SE, Ansehn S. 1988. Rapid susceptibility of *Mycobacterium tuberculosis* by bioluminescence assay of mycobacterial ATP. Antimicrobial Agents and chemotherapy. 32: 1208 - 1212.

78. Normann H, Snyman I, Cohen M. 1996. Indegenous knowledge and its uses in Southern Africa. HSRC Publishers. Pretoria.

79. North RJ, Ryan L, LaCource R. 1999. Growth rate of mycobacteria in mice as an unreliable indicator of mycobacretial virulence. Infection and Immunity. 67: 5483 - 5485.

80. Oleksijew A, Meulbroek J, Ewing P. 1998. *In Vivo* efficacy of ABT-255 against drug sensitive and resistant *M. tuberculosis* strains. Antimicrobial Agents and chemotherapy. 42: 2674-2677.

81. Phillipson JD. 1994. Natural products as drugs. Transactions of the royal society of tropical medicine and hygiene. 88: 17 - 19.

82. Roach DR, Briscoe H, Saunders B. 2001. Secreted Lymphotoxin-α is essential for the control of an intracellular bacterial infection. J. Experimental Medicine. 193: 239 - 246.

83. Rook GWA, Hernandez-Pando R. 1996. The pathogenesis of tuberculosis. Annual reviews. Microbiology. 50: 259 - 284.

84. SaiRam M, Sharma SK, Ilavazhagan G. 1997. Immonomodulatory effects of NIM-76, a volatile fraction from Neem oil. 55:133-139.

85. SaiRam M, Sharma SK, Ilavazhagan G. 1997. Immunomodulatory effects of NIM-76, a volatile fraction from Neem oil. J. Ethnopharmacology. 55: 133 - 139.

86. Sano C, Shimizu T, Tomioka H. 1999. Therapeutic effects of Benzoxazinorifamycin KRM-1648 administered alone or in combination with half -sized secretory leukocyte protease inhibitor or

the nonsteroidal anti-inflammatory drug Diclofenac Sodium against *M.avium* complex infection in mice. Antimicrobial Agents and Chemotherapy. 43: 360-364.

87. Schluger NW, William NR. 1998. The host immune response to tuberculosis. Am J Respir Crit Care Med. 157: 679-691

88. Shen ML, Zhai SK, Chen HL. 1991. Immunomopharmacological effects of polysaccharides from *acanthopanax senticosus* on experimental animals. Int. J. Immunopharmacology. 13: 549 - 554.

89. Shimizu T, Tomioka H, Sato K. 1999. Effects of the Chinese traditional medicine MBST on therapeutic efficacy of a new Benzoxazinorifamycin, KRM-1648, against *M.avium* infection in mice. Antimicrobial Agents and chemotherapy. 43: 514 - 519.

90. Shoen CM, Chase SE, DeStefano M, Harpster TS, Chmielewski AJ, Cynamon MH. 2000. Evaluation of Rifalazil in long-term treatment regimens for tuberculosis in mice. Antimicrobial Agents and chemotherapy. 44: 1458 - 1462.

91. Struillou L, Cohen Y, Lounis N, Bertrand G, Grosset J, Vilde J, Pocidalo J, Perronne C. 1995. Activities of Roxithromycin against *Mycobacterium avium* infections in human macrophages and C57BL/6 mice. Antimicrobial Agents and chemotherapy. 39: 878 - 881.

92. Van Rie A, Warren R, Richardson M. 2000. Classification of drug-resistant tuberculosis in an epidemic area. The Lancet. 356: 22 - 25.

93. Van Wyk BE, Oudshoorn B, Gericke N. 1997. Medicinal plants of South Africa. Briza Publishers. Pretoria.

94. Vickers A, Zollman C. 1999. ABC of complementary medicine: Herbal medicine. British Medical Journal. 319: 1050 - 1053.

95. Vordermeier HM. 1995. T-cell recognition of mycobacterial antigens. European Respiratory journal. 8: 657s – 667s.

96. Quenelle DC, Winchester GA, Staas JK, Barrow WW. 2001. Treatment of tuberculosis using a combination of sustained-release Rifampin-loaded microspheres and oral dosing with Isoniazid. Antimicrobial Agents and chemotherapy. 45: 1637 – 1644.